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ACID PRODUCTION GRAPHICALLY REGISTERED AS AN INDICATOR OF THE VITAL PROCESSES IN THE CULTIVATION OF BACTERIA.

By ALBERT FISCHER.

(From the Albert Fischer Laboratory for Biochemistry and Bacteriology, Copenhagen, Denmark.)

PLATE 54.

(Received for publication, December 5, 1917.)

The investigations described below are a continuation of earlier publications[1] concerning acid production by bacteria of the *coli* group. In the previous investigations the typical acid curve for *Bacillus coli* was determined by titrating at regular intervals the acid produced in the course of *coli* cultivation. But this method was unsatisfactory for several reasons. In the first place, as many *coli* cultures were needed as points were required on the curve. Secondly, there could be no absolute assurance even with the most painstaking care that all the cultures were produced or maintained under constant experimental conditions. And thirdly, when the experiment occupied a longer time, the presence of the observer was required at all hours of the day. A method by which all points necessary for plotting the curve could be obtained from a single culture without depriving the latter of any of its contents or in any other way disturbing the experiment would be more advantageous. Conductivity measurements were attempted as a possible solution of this problem, but, as was to be expected, the presence of strongly dissociated salts such as sodium chloride masked the small changes in conductivity caused by the slightly dissociated acids which were produced.

[1] Fischer, A., and Andersen, E. B., *Centr. Bakteriol., 2te Abt.*, 1912, xxxiii, 289. Fischer, A., *ibid., Ite Abt., Orig.*, 1913, lxix, 474.

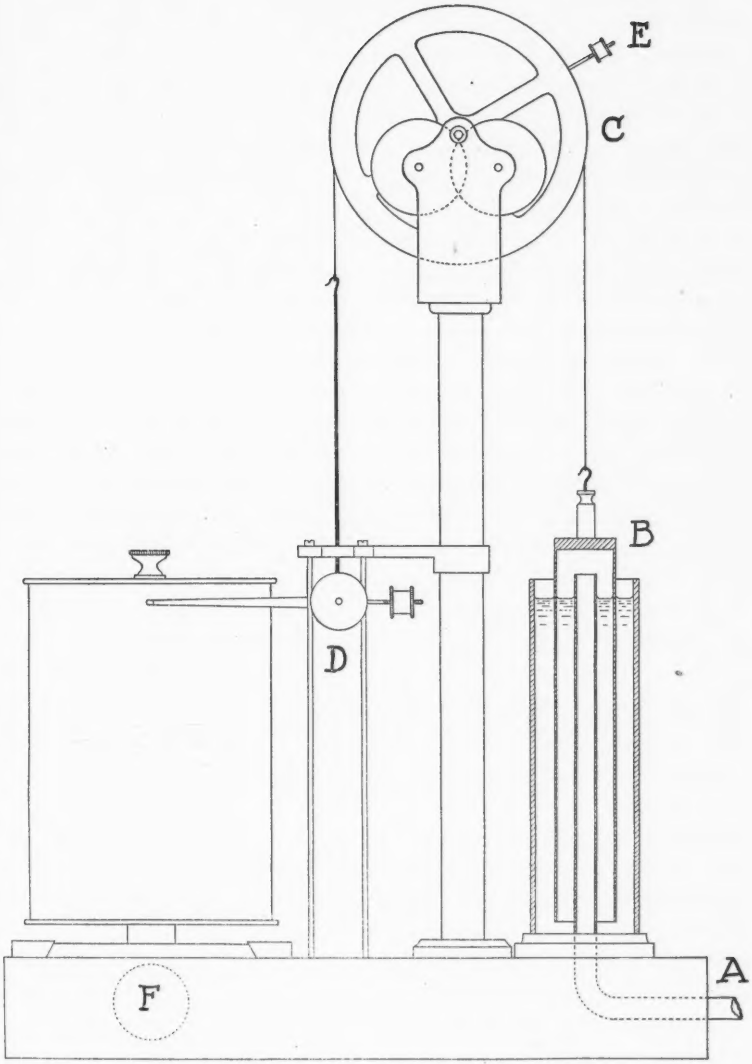
Method.

The method finally adopted was as follows: By the addition of calcium carbonate to the culture, the production of acid was easily followed by the measurement of the carbon dioxide liberated. This in turn was accomplished by a small spirometer, the movements of which were recorded on a rotating cylinder. This method had the following advantages: (1) the acid curve was obtained from the same culture; (2) the process was written in full, whereas formerly only single points were given; (3) constant attention was unnecessary. The procuring of a sensitive self-registering spirometer was a problem in itself. Such an apparatus was formerly utilized for bacteriological experiments and described by Weissenberg.[2]

Great care was taken to make the apparatus used in the present work as sensitive as possible.[3] It is mounted in a heavy mahogany box with glass sides and double doors (Text-fig. 1 and Fig. 1). The apparatus rests on three legs, of which two are separate screws. The carbon dioxide developed from the culture is passed through a glass tube conductor to a tube protruding outside (Text-fig. 1, *A*). This tube is furnished with a three-way stop-cock, not shown on the drawing, which permits communication between the spirometer cylinder and either the culture or the atmosphere. As will be seen from the drawing, the tube protrudes from under a brass cylinder, which is suspended in a glass vessel, containing a 25 per cent glycerol solution. The average diameter of the cylinder is 10 cm. Therefore each 10 cc. of gas at atmospheric pressure will raise the bell 1 cm. By means of a silk thread which is placed over a pulley *C*, the bell is connected with and at the same time partly balanced by the counterweight *D*, which bears the indicator with the writing pen. The pulley is borne upon an upright brass stand, and to reduce friction to a minimum the bearings consist of two pairs of smaller wheels, as shown in the drawing. Since the motion of the pulley wheel is very slow, the inertia of the wheel causes no difficulty. To permit the horizontal adjustment of the axis of the pulley its pointed ends rest in the conically hollowed ends of two steel screws.

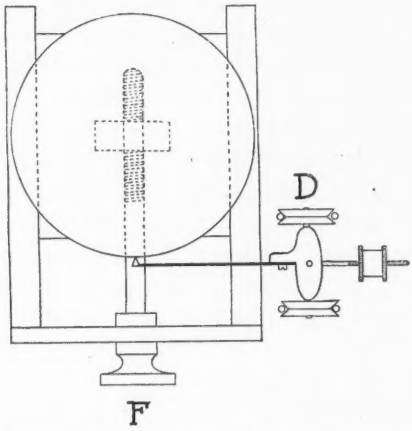
[2] Weissenberg, H., *Centr. Bakteriol., 2te Abt.*, 1902, viii, 370.

[3] The apparatus was made by a mechanical engineer, J. Olsen, Hallinsgade, Copenhagen.



TEXT-FIG. 1. Diagram of the self-registering spirometer used.

As the weight of the cylinder depends upon the extent of immersion in the fluid, this is corrected by a moveable weight *E*, which is placed on the upper part of the pulley in such a position that when the bell is half way out of the fluid it is perpendicularly above the axis of the pulley. If the bell should ascend higher, its weight will increase in proportion to the ascent, or, what amounts to the same thing, in proportion to the angle described by a point on the wheel. Simultaneously the weight moves to the other side of the axis, and by its weight almost always counterbalances the bell, since its moment with regard to the axis of the wheel is proportional to the sine of the angle



TEXT-FIG. 2. Detailed drawing of the rotating cylinder and writing pen.

described. If the diameter of the wheel is large enough so that a given shifting of the bell causes only a small revolution, one may approximately assume the sine of the angle as the angle itself. In this way the varying movements of the weight will almost compensate the varying weight of the bell. If the bell is near its lowest position and the weight on the same side of the axis, the situation is exactly the same. As shown in Text-fig. 2, the counterweight *D* bears two brass wheels which can run against four vertical steel rails. Enough space is allowed between the wheels and the rails so that the wheels do not touch them unless the cylinder presses the pen. The revolving

cylinder has a height corresponding to the bells and a circumference of 48 cm. and revolves by means of a clockwork once in 24 hours. On the roller is placed ordinary millimeter paper. Each hour is represented by 2 cm., while the vertical movement of the indicator of 1 cm. represents a gas evolution of 10 cc. The clock is wound up every 6th day. The whole roller (Text-fig. 2) is placed on a stage which, by means of a screw and bolt, can be moved towards or away from the pen. This is controlled from the outside by means of a pinion wheel *F*. This roller is so adjusted that the wheels on *D* are placed exactly against two of the controlling rails. In order to steady the hands during the motion up and down, the silk thread is not fastened directly to *D* but to a steel hook of considerable length.

For the culture the ordinary tuberculin bulbs were found most suited for the purpose, partly because an ample surface was obtained. Again it was easy to mix by shaking the contents so as to neutralize the acid as quickly as it was formed. Finally, the wide opening was convenient for the introduction of a delivery tube, filling funnel, etc.

To control the conditions of the experiments as much as possible, the culture medium was always prepared in quantities sufficient for a whole series of experiments. Where the experiments did not demand certain changes in the composition of the media, the latter consisted of 2 per cent peptone (Witte's), 0.5 per cent sodium chloride, and 0.5 per cent sugar, generally glucose or lactose. The volume used in each bulb was 100 cc., and to this were always added 100 gm. of calcium carbonate (Kahlbaum). This amount was more than enough to neutralize the largest amount of acid which could be produced by the bacteria in the culture. The bulbs of culture media and calcium carbonate were closed with cotton plugs and autoclaved for half an hour at 120°C., after which the cotton plugs were paraffined and the bulbs placed in a cool dark place ready for use.

For inoculating the cultures precultures were used in order to render the number of bacteria as constant as possible. 24 hours before the beginning of the experiment over 10 cc. of the nutrient medium from a 24 hour agar culture were inoculated, consisting of the following composition: 1 per cent peptone, 0.5 per cent sodium chloride, and 0.5 per cent sugar. In this preliminary culture with a comparatively small amount of nutrient material, the bacteria will have reached

a fairly constant number after 24 hours, even if more or less bacteria had been introduced by means of the platinum needle. The whole of this preculture is added to the tuberculin bulb at the beginning of the experiment. Afterwards the cotton plug is replaced by a sterilized rubber cork, through which passes a glass tube. The rubber tube which leads to the spirograph should be fastened to the glass tube. The bulb is then placed in the incubator and by means of a special shaking apparatus is kept in slow motion so that the culture fluid slowly flows over the layer of calcium carbonate which quickly settles at the bottom.

RESULTS.

There is a marked rise in the curve during the first 2 hours, a result corresponding to the evolution of 20 cc. of gas. This is due to the fact that the air in the tuberculin bulb expands until it reaches the temperature of the incubator. After this the curve becomes horizontal but will rise again, indicating that acid production has begun. In order to insure that the temperature in the incubator had remained constant during the day, a thermograph was placed in the incubator so that a temperature curve was obtained for each curve from the spirograph.

The acid curve produced in this way corresponded exactly to the one found by titration methods on a former occasion. In the first experiments it was important to adjust the composition of the nutrient medium so that the entire process could be made to occur within 24 hours. It was quickly found that the amount of carbohydrate present was the deciding factor. As already stated, in order to adjust the process 0.5 per cent concentration of the specified sugar, as a rule, was used. The process stops as soon as the carbohydrate is consumed. This is shown on the curve as a peculiar sharp break. If more than 0.5 per cent is added, for instance 2 per cent, the production of acid will continue for several days, and the process gradually becomes weaker, finally stopping as a result of the accumulated acids, calcium salts, and cleavage products of the peptone. That this is so may be seen by the fact that the addition of more nourishment does not cause noticeable continuation of the process.

Of the 150 experiments with the spirograph, many were made to test how well the apparatus answered the purpose for which it was designed, and how much could be deduced from the experiments; *i.e.*, how well the experiments could be controlled for obtaining comparative results. It was shown that the changes in pressure and temperature which may take place during a series of experiments are less than the percentage deviation between experiments which are repeated, the deviation amounting at most to about 5 per cent. The temperature in the thermostat can be kept constant, and a changed reading of about 1 per cent will correspond to an alteration in the temperature of the room of 2°C., and 2 per cent to a change in the barometer of 5 mm. That the change in temperature and pressure during 24 hours does not materially influence the curve was shown by making a control test with an uninoculated tuberculin bulb and with the spirometer so arranged that the bell stood half way up from the fluid. In this way almost a horizontal line was obtained.

Before proceeding to the results it should be mentioned that a point on the curve does not conform to the amount of acid produced at that particular moment. This was shown by killing the bacteria when they were vigorously producing acid. It was found that the neutralization of the acid takes place with a certain lag, so that the carbon dioxide evolution takes place for some time after all the bacteria have been killed. For this experiment a strong solution of sublimate (10 cc., 6 per cent) was added to the culture. In this instance and in others where additions to the contents of the bulbs were made during an experiment, the procedure was carried out in such a manner that the curve was not altered by the displacement of air during the filling. A filling funnel is placed in the rubber cork of the tuberculin bulb, where the delivery tube is inserted. When the tap is opened, the contents drop into the bulb and the expelled air will then return to the filling funnel, and consequently not change the pressure in the bulb or spirometer.

These experiments were intended to produce a picture of the growth of the bacteria in a culture; in other words, to make the process visible in a way which can always be reproduced under identical experimental conditions. In a future communication we shall endeavor to give a theoretical explanation of the curves produced. Un-

der constant experimental conditions it has been possible to obtain definite curves. By altering these conditions and by studying the curves obtained, various influences on the life and physiology of the bacteria can be studied.

The investigations which we shall now discuss, but which are as yet not completed, relate especially to the influence of bacteria belonging to the *coli* group on the typical acid curve produced by *Bacillus coli communis*. The reason for employing the *coli* curve was not so much on account of its biology as because it is easy to obtain, and its relation to the carbohydrates presents a special interest, as it can destroy a great number of these with the formation of acid, and is at the same time aggressive towards proteins. Each member of the *coli* group behaves peculiarly towards the different carbohydrates and proteins. Lastly, it is important that the bacteria should be easy to cultivate with a small amount of nutrient material.

Before describing the investigations with mixed infections I shall briefly state the influence of changes in the composition of the nutrient media on the curve.

In order to compare the experiments quantitatively one can derive several numerical relationships from the curves. First, there is the reaction balance, which is ascertained by the combined production of carbonic acid (vital capacity). The process is considered to be completed if at the end of an hour no more than 1 cc. of carbon dioxide is produced. Secondly, there is the speed of reaction, which may be measured by the time consumed for the total carbon dioxide production. This is supplemented by information concerning the speed of the process after the lapse of a certain number of hours (vitality momentum) with the greatest speed attained by the process (optimum vitality). From the numerical data so obtained other curves can be derived, which, for instance, show what influence ascending concentrations of carbohydrates have upon the process. We may, for example, using the amount of sugar as abscissæ and the carbon dioxide production as ordinates, show how lactose, glucose, and peptone in ascending concentration affect the production of carbon dioxide (Table I and Text-figs. 3 to 6). Text-fig. 3 shows the original lactose and glucose curves as registered by the spirograph, reduced about one-ninth.

TABLE I.

Influence of Ascending Concentrations of Carbohydrates on the Acid Curve of B. coli communis.

Glucose.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
per cent	*hrs.*	*cc.*	*hrs.*	*cc.*	*cc.*	
0.3	5.0	35	13.5	5		Process stops abruptly.
0.5	5.0	60	13.5	12		
1.0	4.5	170	29.5	16	13	
2.0	3.5	360	45.5	18	15	
3.0	3.0	540	71.5	19	17	
4.0	3.0	510	98.0	19	16	
5.0	3.5	535	99.0	20	17	

Lactose.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide in 24 hrs.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
per cent	*hrs.*	*cc.*	*hrs.*	*cc.*	*cc.*	
0.3	7.0	35	17.0	9		Process stops abruptly.
0.5	8.0	72		7	6	
1.0	5.5	45		6	5	
2.0	7.5	60		4	4	
3.0	6.0	90		11	10	
4.0	5.0	110		12	10	
5.0	4.0	120		14	12	

Peptone.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
per cent	*hrs.*	*cc.*	*hrs.*	*cc.*	*cc.*	
0.2		1		0	0	
0.5	14	5		0	1	
1.0	6	26		4	3	
1.5	8	28		4	4	

Results with Glucose.

0.5 Per Cent Glucose.—After a lapse of 5 hours the formation of acid begins to be visible on the curve which continues to rise steadily for about 11 hours with a total production of 60 cc. of carbon dioxide. After $13\frac{1}{2}$ hours from the beginning of the experiment a sharp break in the curve is obtained without any previous change in its ascent. From here the curve continues in a horizontal line. This sudden break in the curve, which can take place in less than 3 minutes, can be explained only by the fact that this point marks the disappearance of sugar. This was shown by the fact that sugar could be detected before the break but not afterwards.

1 Per Cent Glucose.—A far greater production of acid is obtained with this sugar concentration. After a lapse of $4\frac{1}{2}$ hours the rise begins and continues for 25 hours, reaching a total production of 170 cc. of carbon dioxide.

2 Per Cent Glucose.—The rise in the curve begins after $3\frac{1}{2}$ hours and continues quite rapidly for 42 hours, reaching a total production of 360 cc. of carbon dioxide. It should be noted that the higher the initial concentration of sugar, the less sharp is the break in the curve. In these instances there is sugar enough and the process does not stop suddenly for lack of nourishment but is gradually retarded because of the inhibiting action of the end-products in the culture.

3 Per Cent Glucose.—After 3 hours the rise begins and continues sharply for $68\frac{1}{2}$ hours with a total production of 540 cc. of carbon dioxide.

4 Per Cent Glucose.—The ascent begins after $3\frac{1}{2}$ hours and continues sharply for about 98 hours, after which the curve breaks, the end being reached after the production of 510 cc. of carbon dioxide.

5 Per Cent Glucose.—After $3\frac{1}{2}$ hours the curve rises sharply for 99 hours and then becomes horizontal. The total production is 535 cc. of carbon dioxide.

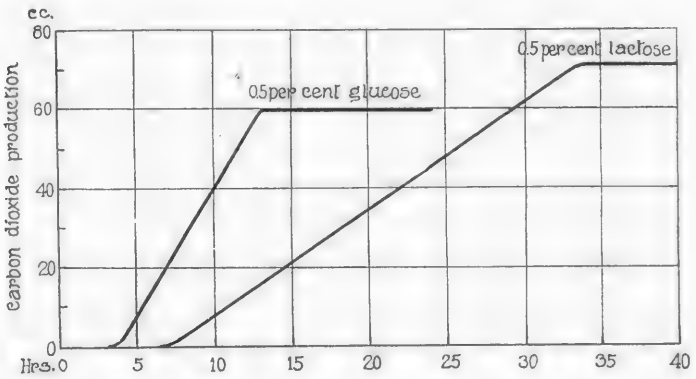
Results with Lactose.

The lactose experiments give only the first part of the curve, as the experiments were interrupted. The curve deviates greatly from the glucose curves. First, it takes somewhat longer for the rise to

begin; secondly, the rise is not so sharp; and thirdly, from the experiments which were completed the duration of carbon dioxide production is greater than in the glucose experiments.

0.3 Per Cent Lactose.—The ascent begins after 7 hours, and lasts about 11 hours, terminating with as sudden a break in the curve as in the case of the curve obtained with the lower concentrations of glucose. The total production was about 35 cc. of carbon dioxide.

0.5 Per Cent Lactose.—The rise begins after about 8 hours and lasts about 27 hours (compare the glucose curve, Text-fig. 3). 72 cc. of carbon dioxide were produced.



TEXT-FIG. 3. The original lactose and glucose curves as registered by the spirograph, reduced about one-ninth.

1 Per Cent Lactose.—After about $5\frac{1}{2}$ hours the ascent proceeded with an average production of 3 cc. per hour and reached about 50 cc. in 24 hours, when the experiment was interrupted.

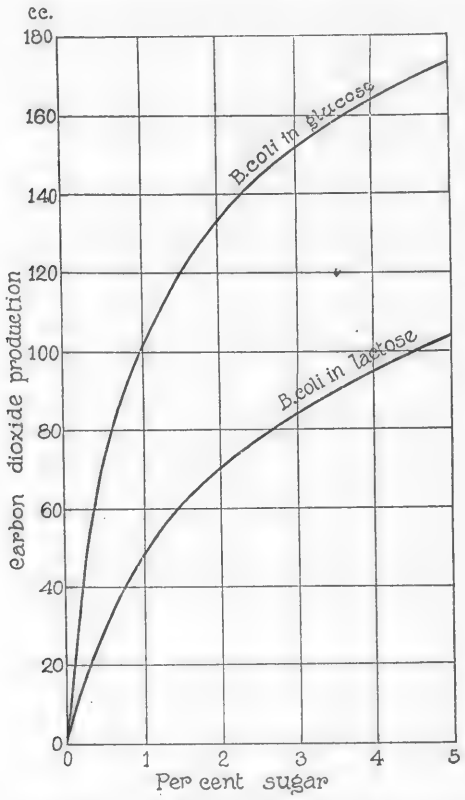
2 Per Cent Lactose.—After about $7\frac{1}{2}$ hours the rise began and gave a total production of 60 cc. in 24 hours.

3 Per Cent Lactose.—After 6 hours the rise began and reached about 90 cc. in 24 hours.

4 Per Cent Lactose.—After about 5 hours the rise began and yielded 110 cc. in 24 hours.

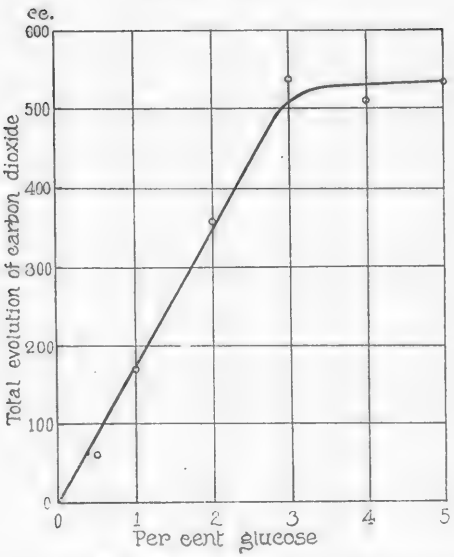
5 Per Cent Lactose.—After 4 hours the rise began and yielded 120 cc. in 24 hours.

It is seen from Table I and the curve (Text-fig. 5) that an ascending concentration of glucose increases the total volume of carbon dioxide produced until about 3 per cent is reached. Greater concentrations of glucose produce no further addition to the total amount of carbon

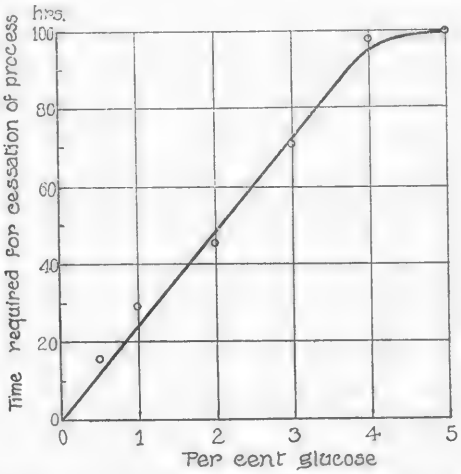


TEXT-FIG. 4. Comparison of the lactose and glucose curves during the first 24 hours.

dioxide formed, whereas the time consumed by the process steadily increases (Text-fig. 6). This indicates that the sugar inhibits the growth of the bacteria. The lactose curve was observed only during the first 24 hours, and when compared with the corresponding period

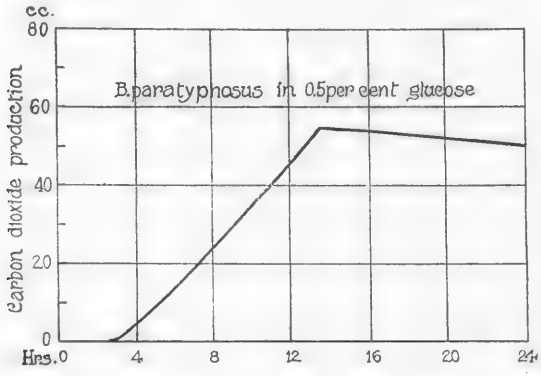


TEXT-FIG. 5. Curve showing the effect of an ascending concentration of glucose upon the total volume of carbon dioxide.



TEXT-FIG. 6. Curve showing the length of time required for the cessation of carbon dioxide production with ascending concentrations of glucose.

of the glucose curves (Text-fig. 4) we perceive, as already mentioned, the longer latent period in the former, the more gradual rise, and, as would appear from the experiments which were completed, a larger total carbon dioxide production. With an increase in the amount of peptone up to 3 per cent the latent period (the time before the rise begins) becomes shorter. When we compare the curve for *Bacillus coli* in 0.5 per cent glucose with the curve for *Bacillus paratyphosus* B also in 0.5 per cent glucose, both curves are seen to be similar, until the point is reached where the process stops. Then while the *coli* curve, as we have seen, continues as a horizontal line, the *para-*



TEXT-FIG. 7. Curve showing the amount of carbon dioxide produced by *B. paratyphosus* B in 0.5 per cent glucose.

typhosus curve (Text-fig. 7) begins to drop (Table II). In the *paratyphosus* curve the ascent begins after 3 hours and after about 12 hours 55 cc. of carbon dioxide have been produced. It then stops suddenly like the *coli* curve, is horizontal for 2 hours, and then drops 5 cc. during a period of 12 hours more. We have not yet been able to explain this. The same curve was found previously by the titration method. By this method it was easier to explain the drop as due to the possible sudden production of ammonia by the bacteria. But here where we are dealing with a method which should show results only in one direction, the case becomes more difficult. At first we naturally thought of the possibility of a leakage of carbonic acid

formed through the connecting tubes. All rubber tubes were therefore replaced with glass tubes, but the curve did not change. That it is a phenomenon connected with the life of the bacteria we have ascertained by killing all the bacteria with sublimate. If ammonia is produced by the bacteria this would be retained in the culture, so that the latter would be able to bind more carbon dioxide than normally.

TABLE II.

B. coli communis and B. paratyphosus B in Mixture.

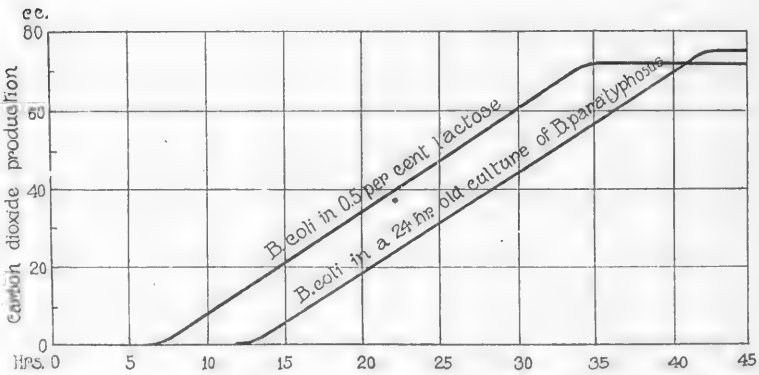
Bacteria.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.	
				After 12 hrs.	After 24 hrs.
Glucose 0.5 per cent.					
	hrs.	*cc.*	*hrs.*	*cc.*	*cc.*
B. coli	3.5	57	13.0	14	0
B. paratyphosus B	3.0	55	13.5	13	0
B. coli added to 24 hr. culture of *paratyphosus*		−16	24.0	−2	−2
B. paratyphosus added to 24 hr. culture of *coli*		−3	24.0	−0.5	0
B. coli + *B. paratyphosus* B	6.0	53	14.5	13.5	−2
Lactose 0.5 per cent.					
B. coli	4.5	76	20.0	11	0
B. paratyphosus B	4.0	−18	24.0	−3	0
B. coli added to 24 hr. culture of *paratyphosus*	12.0	74	44.0	0	3
B. paratyphosus added to 24 hr. culture of *coli*		−14	24.0	−2	−3
B. coli + *B. paratyphosus* B	7.0	48	24.0	6	6

When *Bacillus paratyphosus* is added to lactose media the result is different, since here no acid is produced. But after about 3 hours the curve falls about 20 cc. in the course of 24 hours.

If *Bacillus coli* (Text-fig. 8) is added to a 24 hour *paratyphosus* experiment in lactose, the curve will cease to fall and become a horizontal line in about 14 hours. After this we obtain a steady rise, which, as a rule, lasts a little longer than with the *coli* experiment. In other

words, the latent period for the formation of acid by *coli* is long, which can be explained by the fact that *coli* must overcome the profuse *paratyphosus* growth with its metabolic products. Lack of peptone is partly responsible for this as we have seen that a similarly long latent period was obtained with smaller amounts of peptone. If in like manner we add *coli* to a 24 hour *paratyphosus* growth in glucose, the curve continues to fall. This demonstrates that the decline in the curve is independent of the sugar present, whether glucose or lactose.

We have also proceeded in the opposite direction in order to ascer-



TEXT-FIG. 8. Comparison of the results obtained in lactose with *B. coli* and with a 24 hour *paratyphosus* culture to which *B. coli* has been added.

tain the result when *paratyphosus* is added to a 24 hour *coli* culture. In glucose a fall was noted after the *coli* had stopped producing acid. The same happened in lactose, but the fall was somewhat more abrupt. We finally undertook to determine the effect of adding an equal mixture of *paratyphosus* and *coli* at the beginning of the experiment. In glucose we obtained a curve similar to the *paratyphosus* curve. In lactose we obtained a somewhat longer latent period (7 hours) than with *coli* alone; after that a rise of somewhat longer duration (18 hours) was noted, next a horizontal line (2 to 3 hours), and then the usual fall.

SUMMARY.

We may formulate the results as follows:

1. *Bacillus paratyphosus* in lactose-peptone-water causes a fall in the curve. The explanation of this must be left to later investigations.

2. *Bacillus paratyphosus* in glucose-peptone-water shows at the beginning a rise similar to that produced by *coli*, but a fall in the curve follows.

3. If *Bacillus coli* is added to a 24 hour *paratyphosus* lactose culture a very long latent period is obtained before the formation of acid by *coli* becomes apparent, presumably due to accumulated basic metabolic products.

4. If *Bacillus coli* is added to a 24 hour *paratyphosus* glucose culture the curve is not changed from the normal to any perceptible degree.

5. *Bacillus coli communis* and *Bacillus paratyphosus* in lactose in mixed cultures give a longer latent period than *coli* alone. The first ascending part of the curve is due to acid formation by the *coli* which in the beginning is dominant. Later *paratyphosus* dominates and causes an ultimate decline in the curve.

6. *Bacillus coli communis* and *Bacillus paratyphosus* in glucose in mixed culture behave like *Bacillus paratyphosus* in pure culture.

The purpose of these investigations has been for the present to show the usefulness of the method. It is hoped that by these investigations material of particular interest relating to the biochemical and physiological processes within the bacterial culture will be obtained.

EXPLANATION OF PLATE 54.

FIG. 1. Photograph of the self-registering spirometer used to measure acid production of bacteria.

FIG. 1.

(Fischer: Acid production in cultivation of bacteria.)

EXPERIMENTAL CHEMICAL PNEUMONIA.

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Plates 55 and 56.

(Received for publication, July 28, 1918.)

The employment of solutions of chloramine-T to remove meningococci[1] and other pathogenic microorganisms from the nasopharynx suggested to us its trial in the treatment of experimental pneumonia. Lobar pneumonia was produced in dogs by means of intrabronchial insufflation of a broth culture of a highly virulent Pneumococcus Type I, invariably leading to a fatal termination. The dose was 2.5 cc. of an 18 to 24 hour culture per kilo of body weight. The autopsy on untreated control animals, which usually succumbed within 48 hours, showed a lobar consolidation in the stage of red hepatization of one lobe of the lung, commonly the lower right lobe. The lower left lobe was less frequently affected, and the subcardiac lobe was only occasionally involved, together with one other. The lesions were microscopically typical of experimental lobar pneumonia in the dog.[2] The pneumococcus insufflated was recovered in pure culture from the solid lung area and the blood of the heart.

The next step was to apply the chloramine-T solution to the inoculated lung. The plan was to introduce 5 cc. per kilo of body weight of a 1:10,000 solution intrabronchially 2 hours after the culture was given, and if the animal survived until the next day or longer, to repeat the injection.

Six animals with the experimentally induced pneumonia were given the chloramine-T solution; they were controlled by three untreated dogs. The results can be briefly stated. One animal

[1] Gordon, M. H., and Flack, M., *Brit. Med. J.*, 1916, ii, 673. Dakin, H. D., *ibid.*, 1917, i, 833.

[2] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

received four, another three, and two others two treatments, while two died after the first dose.

Instead of a therapeutic action, the effects of the treatment were rather to intensify the pathological process. The consolidated areas involved more than one lobe and were attended by much greater edema and hemorrhage than were present in the controls. The animal which survived longest (2 days) and received four injections of the chemical, showed pus in both pleural cavities. Moreover, death resulted, as a rule, more quickly in the treated than in the control animals.

This action of the chloramine-T on the infected lungs led us to a study of the effects of this chemical on lungs of normal dogs. The quantity insufflated was 5 cc. per kilo of a 1:10,000 solution of chloramine-T in normal saline solution. The animals were killed with chloroform at intervals varying from 20 hours to 30 days. One dog of the series died on the 2nd day.

After 24 hours the injected lungs showed areas of consolidation. Usually one lobe was affected, but sometimes two or three lobes were involved (Fig. 1). The lesion proved to be bronchopneumonia, attended by marked congestion and edema. The microscope showed that the alveoli contained red corpuscles, epithelial cells, and polymorphonuclear leukocytes. Very little fibrin was present. The capillaries and larger blood vessels were congested and an excess of leukocytes invaded the alveolar walls.

The 48 hour lesion was practically identical with the preceding (Fig. 2). The 72 hour lesions showed less congestion and beginning resolution. The 4 day specimens were more advanced in resolution, while the 7 day specimens were no longer solid and exhibited only decolorized areas to indicate the seat of the previous consolidation. Two dogs were killed with chloroform on the 30th day. They presented a small wedge of organizing (unresolved) pneumonia in the lower right lobe, possibly a residue from the injections. The chloramine-T pneumonia did not cause fever or any appearance of illness in the animals.

A series of experiments was also made with Dakin's hypochlorite of sodium solution (0.48 per cent) in insufflations of 5 cc. per kilo of body weight. The lung lesions produced by insufflation of this

solution were similar to those produced by the chloramine-T solution. They ran about the same course and resolution was apparent on the 3rd day.

SUMMARY AND CONCLUSIONS.

It is obvious from the experiments reported that concentrations of chloramine-T solutions, even more dilute than those which are well borne by the nasopharyngeal mucosa, are injurious to the pulmonary tissue when introduced directly into the bronchi in large volume. The pulmonary lesions produced are of the nature of an extensive bronchopneumonia which progresses during the first 2 days after injection of the chemical and then regresses, to disappear, as a rule, by the 7th day. Similar pulmonary lesions were produced by intrabronchial insufflation of Dakin's solution of hypochlorite of sodium.

These studies taken in conjunction with the earlier ones of Kline and Meltzer[3] in which aleuronat, starch, egg yolk, and lecithin were insufflated into the lungs, show that pulmonary inflammation may be induced by various chemical substances—with the following differences. Aleuronat and starch set up consolidations containing much fibrin, as is the case with virulent pneumococci; egg yolk and lecithin gave lesions with little fibrin, as is the case with avirulent pneumococci. Both series of substances produced lobar pneumonia, while the pulmonary lesions induced by chloramine-T and sodium hypochlorite were of the nature of bronchopneumonia.

The consolidations of the lung produced by chemical substances differ from infectious pulmonary inflammations only in their sterility. These experimental results strongly suggest the view that the anatomical findings in pneumonia represent a part of a mechanism of defense and repair which the animal body creates in its struggle against infection and intoxication.

[3] Kline, B. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1915–16, xiii, 29.

EXPLANATION OF PLATES.

PLATE 55.

FIG. 1. Lungs 24 hours after intrabronchial injection of 1 : 10,000 chloramine-T solution. Dose 5 cc. per kilo of body weight. Right lower lobe swollen, heavy, and solid. Areas of congestion in right upper, and middle lobes. Left lung well aerated.

PLATE 56.

FIG. 2. Section of consolidated lung on the 2nd day after the intrabronchial injection of 1 : 10,000 chloramine-T solution. Dose 5 cc. per kilo of body weight The intraalveolar exudate consists of epithelial cells, polynuclear leukocytes, and erythrocytes. The alveolar walls are infiltrated with polynuclear cells.

FIG. 1.

(Wollstein and Meltzer: Experimental chemical pneumonia.)

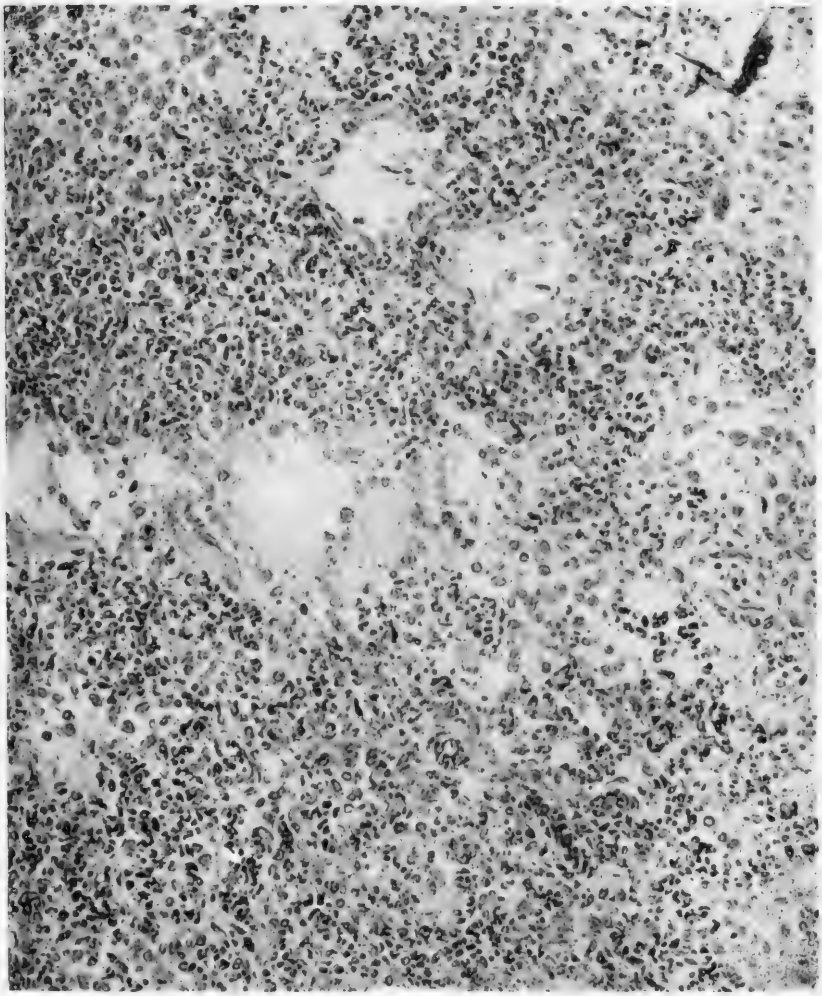

FIG. 2.

(Wollstein and Meltzer: Experimental chemical pneumonia.)

EFFECT OF INTRABRONCHIAL INSUFFLATION OF SOLUTIONS OF SOME INORGANIC SALTS.

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Lamar and Meltzer[1] stated that bronchial insufflation of an isotonic solution of sodium chloride produces no lesion in the lungs. Observations were also made in the course of various experiments carried on by us, which justify the statement that neither hypotonic solutions of sodium chloride nor distilled water causes any pulmonary lesions. In drawing conclusions from these studies the fact should not be forgotten that the posterior part of the lower lobes even of normal dogs frequently shows at autopsy, as previously stated by us,[2] a slight hyperemia when the animals are killed with chloroform.

However, the experience we gathered from the insufflation of sodium hypochlorite caused us to investigate the action of some other inorganic salts in hypotonic and hypertonic solutions. The results are briefly recorded here.

Mercuric Chloride.

Bichloride of mercury was employed in high dilution—1 : 10,000. 5 cc. per kilo of body weight of this solution were insufflated into the bronchi of dogs in the usual manner. At autopsy there was always an unmistakable pulmonary lesion. When the animal was killed after 24 hours the affected lobe of the lung was heavy, dark red in color, and edematous. Microscopically the lesions proved to be largely hemorrhagic in character, and thrombi were present in some of the blood vessels. After 48 hours the hemorrhage and edema were more pronounced, but by the 3rd day the lesion had begun to regress. No bacteria grew in cultures made from the lungs. Obviously the lesion

[1] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

[2] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1913, xvii, 424.

produced by the injection of mercuric chloride directly into the bronchi is not an inflammation; it is merely a hemorrhage which may be so great as to rupture the alveolar walls. This condition is associated with or perhaps caused by thrombosis of the blood vessels.

2 Per Cent Sodium Chloride Solution.

In a series of eight dogs the effect of intrabronchial insufflation of sodium chloride in hypertonic solution (2 per cent equal to about 0.34 M) was tested. 5 cc. of the solution per kilo of body weight were insufflated. Part of the animals were killed 24 hours and others 48 hours after the insufflation. Three of the dogs showed a small area of bronchopneumonia in one lower lobe, while in four others the lungs were practically normal. The eighth animal died soon after the insufflation.

Experiment 1.—December 24, 1917. Black dog, female; weight 5.550 kilos. Received an intrabronchial injection of 28 cc. of sodium chloride, 2 per cent. Killed with chloroform 24 hours later.

Autopsy.—In the right lower lobe there was a wedge-shaped area in the inferior posterior angle measuring 3 by 4 cm. in diameter; it was red, heavy, and swollen.

On section the appearance of the cut surface was that of red bronchopneumonia. Edema and congestion were practically absent from the rest of the lungs.

Microscopic examination proved the lesion to be one of bronchopneumonia, with hemorrhage into some alveoli, and only a small amount of edema.

Experiment 2.—December 24, 1917. Male dog; weight 5.800 kilos. Received intrabronchial insufflation of 30 cc. of sodium chloride, 2 per cent. December 26. Killed with chloroform.

Autopsy.—The right lower lobe contained a wedge in its posterior inferior angle which was not raised, but was dark red in color, and solid.

The cut surface had the appearance of a bronchopneumonia. There was some congestion, but no redness in the other lobes of both lungs.

Microscopic examination showed early bronchopneumonia; the alveoli contained peeled epithelium, red blood cells, granular material, and leukocytes. Other places were more solid, with more leukocytes in the alveoli and infiltrating the walls. The lining cells of the bronchi were normal. There was some periarterial edema.

Experiment 3.—January 3, 1918. Female dog; weight 7.550 kilos. Received intrabronchial insufflation of 38 cc. of sodium chloride, 2 per cent. Killed with chloroform after 24 hours.

Autopsy.—In the posterior inferior angle of the left lower lobe there was a wedge of dark red bronchopneumonia; this area was neither swollen nor heavy; it was

very moist, and the entire lobe was edematous. The right lower lobe contained an area of congestion but no consolidation. Other lobes were normal; there was no pleurisy.

Experiment 4.—January 3, 1918. Male dog; weight 6.300 kilos. Intrabronchial injection of 38 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs were pink and well aerated.

Experiment 5.—January 8, 1918. Male dog; weight 6 kilos. Intrabronchial injection of 30 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs showed no consolidation at any point and no edema; very mild congestion.

Experiment 6.—June 17, 1918. Male dog; weight 8.300 kilos. Intrabronchial injection of 40 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs showed moderate congestion of the right lower lobe. All the other lobes were normal.

Experiment 7.—June 17, 1918. Male dog; weight 9.750 kilos. Intrabronchial injection of 50 cc. of sodium chloride, 2 per cent. Killed with chloroform after 24 hours.

Autopsy.—There was a small amount of congestion in both lungs and in the trachea. Otherwise there were no lesions. Edema was not present.

Experiment 8.—January 18, 1918. Male dog; weight 12.050 kilos. Intrabronchial injection of 60 cc. of sodium chloride, 2 per cent. Found dead after 40 minutes.

Autopsy.—Both lower lobes and the subcardiac lobe were full, heavy, dark red, but translucent. Frothy fluid ran from the trachea.

On section the lungs showed very marked edema, and moderate congestion. There was no bronchitis and no pneumonia. This dog apparently was simply drowned by the intrabronchial injection.

The cultures from the lungs of all the dogs were sterile. Of seven dogs which received intrabronchially the same quantity of a hypertonic solution of sodium chloride, four showed practically normal lungs. The occasional slight congestion observed in these animals may have been due to the use of chloroform in killing them. Three dogs, however, showed lesions. Although the inflammatory area was comparatively small, its bronchopneumonic nature was unmistakable. Attention ought perhaps be called to the fact that these three instances occurred in December and January. It is possible that at this time of the year the lungs are more readily affected by a slight irritant.

As a whole our experiments justify the statement that a hypertonic solution of sodium chloride, administered by intrabronchial injection, causes a comparatively unimportant lesion of the lungs, or none at all.

11 Per Cent Sodium Sulfate Solution.

Five dogs received 5 cc. per kilo of body weight of an 11 per cent solution of sodium sulfate intrabronchially.

Experiment 9.—December 24, 1917. Male dog; weight 6.150 kilos. Received 31 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs were well aerated; there were a few small, dark, congested lobules and practically no edema.

Experiment 10.—December 24, 1917. Female dog; weight 7.300 kilos. Received intrabronchial injection of 38 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

Autopsy.—The lungs were pink and well aerated. A few small congested areas were present but there were no consolidation and no edema.

Experiment 11.—January 3, 1918. Female dog; weight 6.700 kilos. Intrabronchial injection of 40 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

Autopsy.—Only the subcardiac lobe was solid with red bronchopneumonia, and some edema. All the other lobes were well aerated.

Experiment 12.—January 3, 1918. Female dog; weight 7.450 kilos. Injected intrabronchially with 48 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 24 hours.

Autopsy.—The right lower lobe contained a wedge-shaped area of bronchopneumonia, rather gray and very moist; there was much edema. No lesion in the other lobes.

Experiment 13.—January 8, 1918. Male dog; weight 8.600 kilos. Intrabronchial injection of 45 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 24 hours.

Autopsy.—The subcardiac lobe was translucent, dark red in color.

On section, it showed bronchopneumonia with plugs of mucus in the bronchi. In the other lobes there was a moderate amount of congestion and edema but no consolidation. A general bronchitis was present. The animal undoubtedly suffered from distemper.

In the first two experiments of this series the intrabronchial injection of a distinctly hypertonic solution of sodium sulfate caused no lesion whatsoever. The last experiment was an instance of distemper. It is not impossible that the dogs in Experiments 11 and 12 were not

normal when the experiment was begun. At any rate, the experiments seem to justify the general statement that an intrabronchial injection of a hypertonic solution of sodium sulfate, like a similar solution of sodium chloride, causes either no pulmonary lesion at all or only an insignificant one.

Magnesium Salts.

A few experiments were made with intrabronchial insufflation of magnesium salts. It should be borne in mind that the intrabronchial use of these salts is fraught with danger. In the first place, it may cause deep anesthesia and paralysis of respiration. The latter could, however, be obviated by intravenous or intramuscular injection of calcium chloride. More serious is the paralytic effect which the magnesium salt may exert upon the heart. Coming directly in contact with the capillaries of the lung, the solution of magnesium salts, without previously being diluted by the blood of the right ventricle, enters the left ventricle and its coronary artery and may thus cause a fatal effect upon the activity of the left ventricle. Two experiments were made with magnesium sulfate and four with magnesium chloride.

Experiment 14. Magnesium Sulfate.—September 12, 1917. 5 cc. per kilo of body weight of hydrated magnesium sulfate, containing seven molecules each of water of crystallization, were introduced intrabronchially. The animal was etherized by intratracheal insufflation and at 10.12 a.m., a cannula was introduced into the jugular vein, to be ready for an injection of calcium chloride. 12 m. 50 cc. of a 6 per cent solution of magnesium sulfate were introduced intrabronchially. 12.27 p.m. The animal was awake but not struggling; placed on the floor. 12.54 p.m. Still recumbent but the head was up. The cannula was removed and the wound stitched. 5 p.m. Still lying down; coughing; temperature 40.2°C. September 13, 9 a.m. No coughing; quiet; temperature 39.3°C. September 14, 9 a.m. Temperature 39.5°C. Condition improved. 11.30 a.m. Killed with chloroform (48 hours after intrabronchial injection of the salt).

Autopsy.—The posterior part of the right lower lobe was half solid, red, swol en, moderately congested, not very heavy, not friable.

The cut surface showed lobular pneumonia, which was confirmed by microscopic examination.

Experiment 15. Magnesium Sulfate.—January 8, 1918. Female dog; weight 10.650 kilos. Injected intrabronchially with 56 cc. of magnesium sulfate, 8.38 per cent, followed by an intramuscular injection in the right thigh of 10 cc. of calcium chloride, 2.5 per cent. January 9, 9 a.m. Animal quiet, lying down;

temperature 39.2°C. January 10, 9 a.m. Temperature 39.5°C.; quiet. 10.30 a.m. Killed with chloroform (about 24 hours after injection of the solution of magnesium sulfate).

Autopsy.—Right lower lobe heavy, swollen, edematous, and congested; not very solid.

The cut surface was mottled with bronchopneumonic areas, especially in the upper part of the posterior border. A moderate amount of congestion was present in the right upper and middle lobes.

Experiment 16. Magnesium Chloride.—January 8, 1918. Male dog; weight 6.850 kilos. 11.44 a.m. Injected intrabronchially with 35 cc. of magnesium chloride, 6.92 per cent (about 5 cc. per kilo), followed by an injection into the right thigh of 10 cc. of calcium chloride, 2.5 per cent. 4 p.m. Temperature 40.1°C. January 9, 9 a.m. Temperature 38.5°C. Animal lively. 10.30 a.m. Killed with chloroform (about 24 hours after injection of magnesium chloride solution).

Autopsy.—The apex of the right upper lobe was solid and swollen; on section very dark areas of hemorrhage alternated with pale red areas of bronchopneumonia. The lower lobe was less solid than the upper; on section it was mottled with areas of bronchopneumonia; there were marked edema and moderate congestion.

Experiment 17. Magnesium Chloride.—June 24, 1918. Female dog; weight 8.150 kilos. Injected intrabronchially with 33 cc. of magnesium chloride, 6 per cent (about 4 cc. per kilo). No rise of temperature followed. The dog was killed with chloroform after 24 hours.

Autopsy.—The right lower lobe contained a firm, dark blue area in the posterior inferior angle, 3 to 4 cm. in diameter. On section, this showed bronchopneumonia with marked edema; the area was red in color and congested moderately. A small amount of frothy fluid in the trachea and the main bronchi.

Experiment 18. Magnesium Chloride.—June 24, 1918. Female dog; weight 14.050 kilos. Given an intrabronchial injection of 56 cc. of magnesium chloride solution, 6 per cent (about 4 cc. per kilo). No rise in temperature followed. June 26, 11 a.m. Animal normal; killed with chloroform (48 hours after injection of magnesium chloride).

Autopsy.—There was a small superficial area of congestion in the left lower lobe about the middle of the posterior border. All the other lobes were well aerated, slightly congested, and not edematous. No frothy fluid in the trachea.

Cultures made from the heart and lungs of the animals intrabronchially injected with magnesium salts showed no growth.

Two more experiments with insufflation of magnesium chloride were made, but they do not belong to this series. They are recorded merely to show the danger of an intrabronchial insufflation of a concentrated solution of a magnesium salt. Through an error a 6 per cent solution of magnesium chloride was prepared from a dehydrated

salt. Without knowing this fact the solution was used on two dogs, giving 4 cc. per kilo. The first dog died a few minutes after the insufflation. The history of the second dog follows:

Experiment 19.—Male dog; weight 12.950 kilos. June 17, 1918, 2 p.m. An intrabronchial injection of 50 cc. of magnesium chloride, about 14 per cent (about 4 cc. per kilo), was given. 2.30 p.m. There was no spontaneous respiration and intratracheal insufflation was started. Froth was emitted from the nose and mouth. Given 30 cc. of calcium chloride, 2.5 per cent, into the muscles of the right thigh. 2.55 p.m. Spontaneous respirations appeared and became regular; lid reflex fair. The insufflation was discontinued, but the tube was left in the trachea for 30 minutes, then removed. 5 p.m. Temperature 37.3°C.; lying down, sick. June 18, 9 a.m. Temperature 39.9°C. 5.45 p.m. Found dead.

Autopsy.—Performed next day. No consolidation in the lungs. The right lower lobe was slightly decolorized and full in the posterior portion. On section, it was congested. Frothy fluid was present in the bronchi. The left lung and the trachea contained no fluid. The 30 cc. of calcium given intrabronchially may have prevented for a time the fatal effect of the magnesium.

From the experiments given above it would seem that magnesium salts are more liable to produce (moderate) pulmonary lesions than sodium salts and that magnesium sulfate may be more effective than magnesium chloride. However, we must bear in mind that the number of experiments in these series is too small to draw definite conclusions from them. For the same reason we shall not attempt to discuss whether this effect of magnesium sulfate upon the lung could be connected with the observation of Gates[3] on the production of hyaline casts by intravenous injections of magnesium sulfate.

SUMMARY.

Intrabronchial injections of isotonic as well as of hypotonic solutions of sodium chloride or even of distilled water cause no pulmonary lesions.

Intrabronchial injections of mercuric chloride even in a dilution of 1 : 10,000 cause a marked pulmonary lesion. The lesion is not of an inflammatory character; it consists of congestion, formation of thrombi, and hemorrhage.

[3] Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 102. See also Gates, F. L., and Meltzer, S. J., *ibid.*, 167.

Intrabronchial injections of hypertonic solutions of sodium chloride as well as of sodium sulfate cause, in most instances, no lesions whatsoever. In a smaller number of cases in which moderate lesions were present they may have been due either to a previous infection (distemper) or to some predisposing cause (winter months).

Intrabronchial injection of magnesium salts apparently tends to cause moderate pulmonary lesions (bronchopneumonia). This seems especially true of magnesium sulfate.

THE SPIROCHETAL FLORA OF THE NORMAL FEMALE GENITALIA.

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PLATE 57.

(Received for publication, June 27, 1918.)

The present paper is a sequel to a recently published[1] study of the spirochetal flora of the male genitalia. The subject has been taken up, not because there was any reason for supposing the spirochetal flora of the female genitalia to be different from that of the male, but because of the relative infrequency of any special reference to the former in literature, and in order to obtain more accurate knowledge concerning it. Except for the occurrence of the well known *Spironema refringens* (Schaudinn and Hoffmann[2]) in both male and female genitalia, practically nothing is known about the spiral organisms in the normal female genitalia.

On anatomic and physiologic grounds the female genitalia undoubtedly afford more favorable conditions for the existence of these saprophytic spirochetes, and the results of examinations of the smegma, films, and washings of the genital mucous membranes of normal adult females conclusively demonstrate that the number of spirochetes of the female is much greater than that of the adult male, although the varieties present appear to be identical in both. As in the male smegma *Treponema calligyrum* is encountered here also as the predominating variety in the majority of specimens, while *Treponema minutum* is present almost constantly but in fewer numbers. On the other hand, the coarse *Spironema refringens* has been less frequently met with here than in the male smegma. The finding may have been accidental but is recorded here for future reference.

[1] Noguchi, H., The spirochetal flora of the normal male genitalia, *J. Exp. Med.*, 1918, xxvii, 667.

[2] Schaudinn, F., and Hoffmann, E., *Arb. k. Gsndhtsamte.*, 1905, xxii, 527.

The morphologic and cultural characteristics of these three types were described in a previous publication.[1] In Figs. 1 to 8 *calligyrum*, *minutum*, and *refringens* are easily distinguished. The organisms stained by Fontana's method (Figs. 1 to 3) appear more distinctly than those stained by the mordant gentian violet method (Figs. 4 to 8), but in either case there is no difficulty in recognizing the types. Here, as with the male smegma, the use of the dark-field microscope is important in order to avoid confusion of the organisms belonging to the *Leptospira* group with those normally present.

Examination of the spirochetal flora of female children up to the age of 2 years showed that the varieties present are similar to those found in the adult, differing from the latter only in being less numerous.

CONCLUSION.

The spirochetal flora of normal female genitalia is similar to that of the male and consists of *Treponema calligyrum*, *Treponema minutum*, and *Spironema refringens*. The types are present in varying proportions, but the *calligyrum* usually predominates, and the *refringens* is the least frequent. The female genitalia are much richer generally in the number of spirochetes than the male.

EXPLANATION OF PLATE 57.

Magnification, × 1,000.

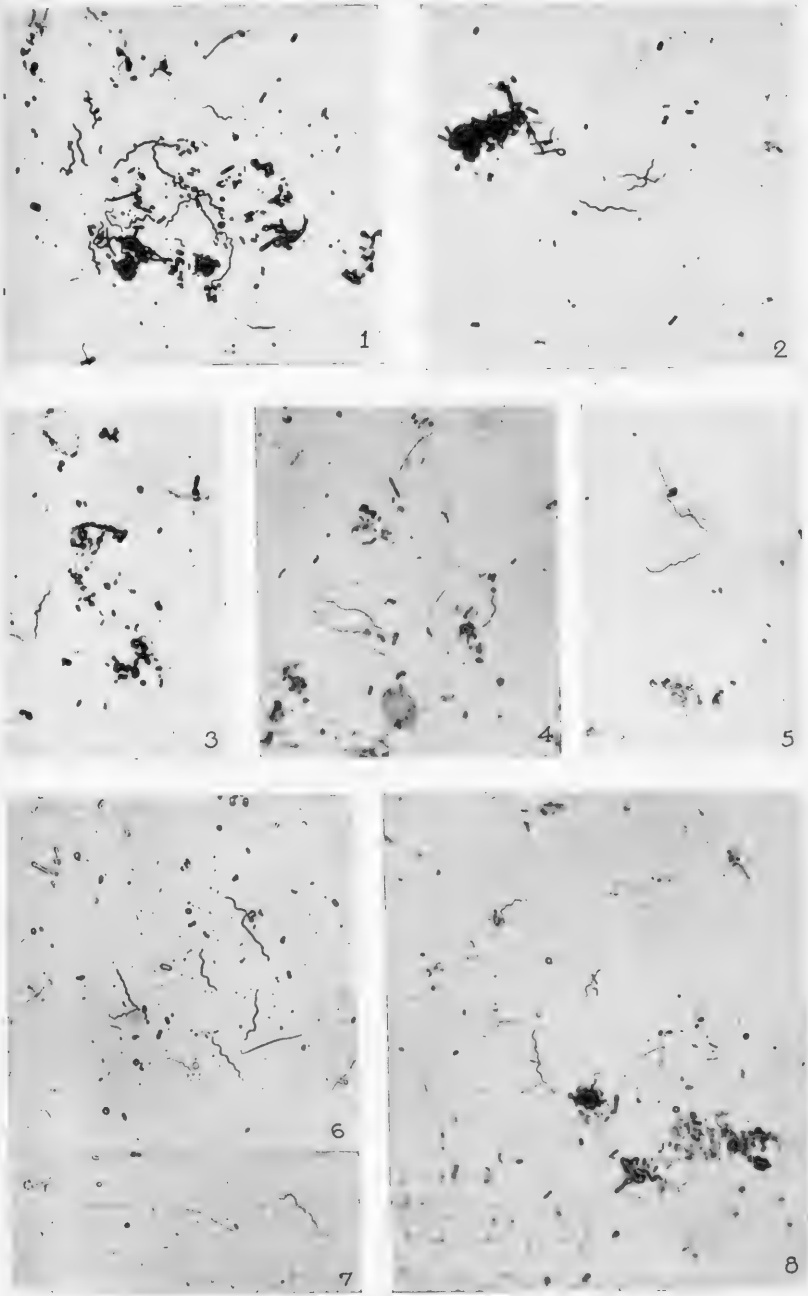
FIGS. 1 to 3. Films from female smegmas, showing *Treponema calligyrum* and *Treponema minutum*.

FIG. 4. Several specimens of *Treponema calligyrum* and a few of *Spironema refringens*.

FIG. 5. Several specimens of *Treponema calligyrum* and one of *Treponema minutum*.

FIG. 6. The three types.

FIGS. 7 and 8. *Treponema calligyrum* and *Treponema minutum*.



(Noguchi and Kaliski: Spirochetal flora of normal female genitalia.)

A COMPARATIVE STUDY OF EXPERIMENTAL PROPHYLACTIC INOCULATION AGAINST LEPTOSPIRA ICTEROHÆMORRHAGIÆ.

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Ido, Hoki, Ito, and Wani[1] first demonstrated that when guinea pigs are treated with three successive injections of killed cultures of *Leptospira icterohæmorrhagiæ* at 5 day intervals they become refractory to a subsequent infection, undertaken 10 days later, with a virulent culture or with liver emulsions of infected guinea pigs. After about 17 to 19 days the serum of these animals contained a small amount of immune bodies when tested by the method of Pfeiffer. From this experiment the conclusion was drawn that the degree of immunity induced in these animals is comparable with that which develops in the serum of patients on the 8th day of disease, in the stage when the infective organisms have already disappeared from the peripheral blood, as shown by negative results of animal inoculations with the blood. The immunity is incomplete, but sufficiently strong to protect the animals from the infection. These writers found also that the serum of six persons inoculated with three successive doses of highly concentrated vaccine (carbolized emulsion of the pure culture) for the purpose of prophylaxis contained a demonstrable amount of immune bodies when tested by Pfeiffer's method 10 days after the last inoculation, but when given to unvaccinated guinea pigs the serum failed to protect the animals against a simultaneous infection. There was considerable difference, however, between the course of the disease in the animals receiving the serum from vaccinated persons and those receiving normal human serum, the former outliving the controls by as many as 10 days, the number of spirochetes being fewer, and the icterohemorrhagic lesions less marked.

Ito and Matsuzaki[2] recommended for the purpose of protective vaccination the use of the blood gelatin culture of *Leptospira icterohæmorrhagiæ*. The culture was killed by heating it to 60°C. for 30 minutes on 3 successive days. The killed culture was toxic when given intravenously, and one of the samples killed guinea pigs, in a dose of 0.5 cc. or more per 100 gm. of body weight, within a few minutes. A single subcutaneous injection of 0.05 cc. of another preparation of the killed cul-

[1] Ido, Y., Hoki, R., Ito, H., and Wani, H., The prophylaxis of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 471.

[2] Ito, T., and Matsuzaki, H., Prophylactic vaccination, serotherapy, and chemotherapy in Weil's disease (in Japanese), *J. Chiba Med. College*, 1916, Nos. 83 and 84.

ture, the minimum lethal dose of which was 1.5 cc. per 100 gm. of body weight, was found to be sufficient to confer upon the animal a complete immunity against a subsequent infection after 2 weeks, although there was no protection at the end of the 1st week. Ito and Matsuzaki also prepared a pulverized vaccine from a desiccated blood gelatin culture of the *icterohæmorrhagiæ* and found that it not only retained its immunizing quality but possessed the advantage over the liquid vaccine of being nearly ten times more concentrated. For protective vaccination they used a saline emulsion of the dried culture. They calculated the quantity necessary for a man of 50 kilos body weight to be 25 to 30 cc. of the liquid and 2.5 to 3 gm. of the dried material. The serum from the vaccinated animals contained the specific immune bodies demonstrable by Pfeiffer's procedure and also *in vitro* (Sabritschewsky's phenomenon).

From the experiments of Ido, Hoki, and others on the one hand, and those of Ito and Matsuzaki on the other, it is evident that the guinea pig, which is perhaps the animal most susceptible to infection by *Leptospira icterohæmorrhagiæ*, can be rendered actively immune by a single injection or by multiple injections of devitalized cultures. The question arises as to whether the immunity thus induced with one strain will protect the animal equally well against the other strains. The present work was undertaken to determine the relation of one strain to another by means of cross-immunity reactions among the strains isolated in Japan, America, and Europe.

Method.

For the purpose of protective inoculation guinea pigs weighing about 400 gm. were selected. The emulsions of *Leptospira icterohæmorrhagiæ* were prepared from pure cultures of different strains grown on the rabbit serum Ringer medium (semisolid below and liquid above[3]) at 26°C. for from 3 to 6 weeks. The liquid portion of the culture, showing 20 to 30 organisms per field ($\frac{1}{12}$ oil immersion and Leitz ocular 3), was gathered from a number of tubes and, after immobilization of the organisms by means of 0.4 per cent phenol (determined by the dark-field microscope), was centrifuged for 2 hours at the rate of 4,000 revolutions per minute; the deposit was carefully separated from the supernatant fluid and resuspended in one-fifth

[3] Noguchi, H., Further study on the cultural conditions of *Leptospira* (*Spirochæta*) *icterohæmorrhagiæ*, *J. Exp. Med.*, 1918, xxvii, 593.

of the original volume. The emulsion thus concentrated contained a great number of the organisms and was preserved under a layer of toluene for 1 week in the refrigerator (6°C.) before use. The emulsion was then tested by culture in order to be certain that there were no live organisms left.

Emulsions were prepared from two American strains isolated from wild rats caught in the vicinity of New York, one European, and one Japanese strain,[4] and the inoculations were made subcutaneously. In the first group of animals 0.5 cc., in the second 0.05 cc., and in the third 0.005 cc. of the emulsion was given three times at intervals of 5 days. Except in a few instances the animals showed no rise in temperature after the inoculation. As a whole they stood the inoculations well, though a number were lost through secondary infection. Some lost weight during the vaccination period.

Tests for active immunity against the homologous and heterologous strains were made at the end of 2, 4, and 8 weeks after the last inoculation. The quantities used for the injection intraperitoneally into the vaccinated and control (unvaccinated) animals were those containing at least several minimum lethal doses of each strain. For example, in the case of American Strain 1, 0.0005 cc. was used, American Strain 2, 0.005 cc., American Strain 3, 0.05 cc., Japanese, 0.00005 cc., and European, 0.005 cc. of 3 week cultures on the rabbit serum Ringer medium at 26°C. Two control animals accompanied each series of experiments, and they always died of the typical infection within 5 to 7 days.

Notwithstanding the care which they received, some of the vaccinated guinea pigs died of secondary infection, or in a few instances of marasmus, before the experiments were completed.

RESULTS.

The results of the tests are briefly summarized in Tables I to IV.

Table I shows that the guinea pigs receiving three successive injections of 0.05 and 0.5 cc. of the concentrated vaccine of American Strain 1 were, with two exceptions, protected against a subsequent

[4] Noguchi, H., *Spirochæta icterohæmorrhagiæ* in American wild rats and its relation to the Japanese and European strains, *J. Exp. Med.*, 1917, xxv, 755.

TABLE I.

Vaccine Made from American Strain 1.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
American Strain 1	Survived.	Survived.	Died in 7 days.	Died in 6 days.	Survived.	—	Survived.	Died in 6 days.	—	Survived.	Survived.	Died in 7 days.
" " 2	"	Died in 8 days.	Died in 9 days.	Died in 6 days.	"	Survived.	Died in 7 days.	Died in 6 days.	Survived.	"	Died in 8 days.	Died in 6 days.
" " 3	"	Survived.	Died in 8 days.	Died in 5 days.	—*	"	Survived.	Died in 6 days.	"	—	Survived.	Died in 7 days.
Japanese strain.	"	"	Died in 8 days.	Died in 6 days.	Survived.	"	Died in 11 days.	Died in 5 days.	"	Survived.	Died in 7 days.	Died in 5 days.
European "	"	Died in 8 days.	Died in 8 days.	Died in 7 days.	"	"	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.

*— indicates that the animal succumbed to secondary infection.

TABLE II.

Vaccine Made from American Strain 2.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
American Strain 2	Survived.	Survived.	Died in 7 days.	Died in 6 days.	Survived.	Survived.	Died in 9 days.	Died in 7 days.	--	Survived.	Died in 9 days.	Died in 6 days.
" " 1	"	"	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	Survived.	Died in 13 days.	—	Died in 6 days.
" " 3	"	"	Died in 9 days.	Died in 7 days.	"	"	Died in 7 days.	Died in 7 days.	"	—	Died in 7 days.	Died in 7 days.
Japanese strain.	"	Died in 10 days.	Died in 7 days.	Died in 5 days.	"	—	Died in 8 days.	Died in 5 days.	"	Died in 10 days.	Died in 6 days.	Died in 5 days.
European "	"	Survived.	Died in 8 days.	Died in 7 days.	"	Survived.	Died in 8 days.	Died in 6 days.	"	Survived.	Died in 8 days.	Died in 6 days.

TABLE III.

Vaccine Made from Japanese Strain.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
Japanese strain.	Survived.	Survived.	Died in 11 days.	Died in 5 days.	Survived.	Survived.	Survived.	Died in 5 days.	Survived.	—	Survived.	Died in 5 days.
American Strain 1	"	"	—	Died in 6 days.	—	"	Died in 9 days.	Died in 6 days.	"	Survived.	Died in 8 days.	Died in 6 days.
" " 2	"	—	Died in 9 days.	Died in 7 days.	Survived.	"	Died in 10 days.	Died in 7 days.	—	Died in 9 days.	Died in 11 days.	Died in 7 days.
" " 3	"	Survived.	Died in 7 days.	Died in 6 days.	"	"	Died in 12 days.	Died in 6 days.	Survived.	Survived.	Died in 13 days.	Died in 6 days.
European strain.	"	Died in 8 days.	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	"	Died in 12 days.	Died in 8 days.	Died in 6 days.

TABLE IV.

Vaccine Made from European Strain.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
European strain.	Survived.	Survived.	Died in 8 days.	Died in 5 days.	Survived.	Survived.	Survived.	Died in 6 days.	Survived.	Survived.	Died in 12 days.	Died in 6 days.
American Strain 1	"	Died in 12 days.	Died in 8 days.	Died in 6 days.	—	"	Died in 8 days.	Died in 6 days.	"	Died in 9 days.	Died in 8 days.	Died in 6 days.
" " 2	"	Survived.	Died in 9 days.	Died in 6 days.	Survived.	"	Died in 13 days.	Died in 6 days.	—	Survived.	Died in 9 days.	Died in 6 days.
" " 3	"	"	Died in 13 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	Survived.	"	—	Died in 7 days.
Japanese strain.	"	Died in 8 days.	Died in 6 days.	Died in 5 days.	"	—	Died in 6 days.	Died in 5 days.	"	Died in 8 days.	Died in 6 days.	Died in 5 days.

infection undertaken 2, 4, and 8 weeks after the last inoculation. One exception occurred in the case of American Strain 2 and one in the case of the European strain, both animals succumbing to the infection 1 to 2 days later than their respective controls. On the other hand, three injections of 0.005 cc. of the same vaccine protected the animals against the same strain as well as against American Strain 3, but failed to produce complete immunity against the other three heterologous strains.

Table II gives the results obtained with the guinea pigs vaccinated with American Strain 2. The protection was complete in the group receiving 0.05 and 0.5 cc., except when tested against the Japanese strain and American Strain 1, in which instances death was delayed but not prevented. There was no resistance against any strain when the dose of the vaccine was 0.005 cc.

In the series reported in Table III, the highest degree of immunity is seen to have been developed against the Japanese strain, which was the one used as the vaccine in this series. It was the only strain which failed to kill guinea pigs that received injections of the smallest amount of the vaccine (0.005 cc.). Protection was weakest against American Strain 2 and the European strain. Three injections of 0.5 cc. in succession, however, conferred complete immunity against all the strains tested.

A similar relation exists between the homologous and heterologous strains of the next series (Table IV), the protection being most marked against the same organism. All the guinea pigs vaccinated with 0.5 cc. of any one strain resisted infection with that and all other strains used in the present study.

While these results do not furnish basis enough to permit division of the various strains into groups or definite types, yet the general indication is that there is a closer affinity among some strains than others. For example, American Strains 1 and 3 seem to be closely allied, American Strain 2 being nearer to the European strain. The Japanese strain stands between the two groups and much nearer that formed by American Strains 1 and 3.

With respect to the duration of the immunity thus established, it was found that at the end of 8 weeks there was not an appreciable diminution of the immunity, although of the animals receiving smaller amounts of the vaccine a slightly larger number survived when tested at the end of 4 than at the end of 2 or 8 weeks.

SUMMARY AND CONCLUSIONS.

Guinea pigs were inoculated with suspensions of *Leptospira icterohæmorrhagiæ* obtained from pure cultures of several different strains, in order to determine whether or not an active immunity against a subsequent infection with virulent organisms would develop in the vaccinated animals. The experiments were so arranged as to make possible a determination of the existence of immunity against homologous strains as well as against the strains not employed as vaccine, and a brief quantitive estimation of the degree and duration of the immunity in relation to the quantities of the vaccines inoculated. Following the general rule of prophylactic inoculations with various pathogenic organisms, the inoculations were repeated subcutaneously on three consecutive occasions at intervals of 5 days. With respect to the amounts of vaccine, the experiments were divided into three groups for each vaccine, one group receiving three doses of 0.5 cc., the second three of 0.05 cc., and the third three of 0.005 cc. Four different strains were employed as vaccines, American Strain 1, American Strain 2, and one each of the Japanese and the European strains.

The determination of the development, degree, and duration of the immunity was made by inoculating intraperitoneally several minimum lethal doses of each of the five following strains: American Strains 1, 2, and 3, the Japanese, and the European strains. The virulence of the different strains varied considerably, the strongest being the Japanese strain, which killed the guinea pig in a dose of 0.00001 cc., and the weakest American Strain 3, the minimum lethal dose of which was as large as 0.01 cc.

The vaccinated guinea pigs were tested for immunity at the end of 2, 4, and 8 weeks after the last inoculation.

The results obtained show that three successive inoculations of 0.5 cc. of the emulsions of killed cultures of *Leptospira icterohæmorrhagiæ* into guinea pigs rendered them completely resistant to a subsequent infection with the virulent cultures of both homologous and heterologous strains. With 0.05 cc. the protection was not so general, the animals succumbing to an experimental infection with some heterologous strains while resisting the homologous and other heterologous strains. The animals which were vaccinated with 0.005 cc. survived

the infection experiments with the homologous strains in the case of American Strain 1 and the Japanese strain, but they were not protected against any other strains. The vaccines of other strains were unable to immunize the guinea pigs so highly even against their homologous strains, when the amount of each inoculation was only 0.005 cc., but 0.05 cc. conferred complete protection against the same strains. It may be concluded, therefore, that when a sufficient quantity of killed cultures of *Leptospira icterohæmorrhagiæ* is given, the guinea pigs will become immune to all strains of the same organism, but that smaller quantities may protect them against homologous but not against heterologous strains. A close analysis reveals the existence of group or type affinities among different strains which can be brought out by immunizing the animals with smaller quantities of killed cultures. In the present series of experiments American Strains 1 and 3 form one group, American Strain 2 and the European strain another, and the Japanese strain a third, which is also closely allied to the first group.

In order to insure universal immunity it is wise to employ as many group or type cultures as possible in the preparation of vaccines, a polyvalent vaccine being recommended. It is not improbable that the strain recently encountered in Lorient, France,[5-8] is an unusually deviated type of *Leptospira icterohæmorrhagiæ*, and that if successfully cultivated and used as vaccine in sufficient amount it might protect the animals against other strains of the same organism.

The active immunity induced in the vaccinated guinea pigs was found to persist for at least 8 weeks after the last inoculation. It will no doubt last for a much longer period.

[5] Manine, Cristau, and Plazy, *Compt. rend. Soc. biol.*, 1917, lxxx, 531.

[6] Manine and Cristau, *Bull. et mém. Soc. méd. hôp. Paris*, 1917, xli, series 3, 977, 1045.

[7] Pettit, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 774.

[8] Pettit, A., *Compt. rend. Soc. biol.*, 1918, lxxxi, 48.

A SPECIFIC POISON IN THE LIVER EXTRACTS OF RABBITS INOCULATED WITH TYPHOID AND PRODIGIOSUS BACILLI INTRAVENOUSLY.

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The work of Parker and Franke[1] shows that when rabbits are inoculated with typhoid bacilli intravenously, there is not enough difference in either the killing off or localization of the bacteria in the organs, of normal and immune animals, to account for the difference in the latter's resistance to the infection. The experiments seem to indicate that the rabbits immune to typhoid bacilli might owe their resistance to the power to neutralize or destroy the poisons as they were being formed from the bacteria while normal animals either lacked this power or possessed it imperfectly. The liver, on account of its size and because it takes up so many more typhoid bacilli per gram of weight than any other organ, was thought of as operating in this connection. Indeed, the liver might not only be the main seat of the poison formation but might act as a detoxicant in the immune animal, but not in the normal animal. The work of Manwaring[2] and Weil[3] on the liver in anaphylaxis in dogs bears on this point. It was with the purpose of investigating this question that the experiments to follow were performed.

Dold,[4] Loeb,[5] and Dold and Ogata,[6] and others have studied the toxicity of aqueous extracts of normal organs when given intravenously. Their technique was practically the same as that used in our experiments except that they did not weigh the organs. Although Dold states that all normal rabbit organs (lung, spleen, liver, kidney, brain, and muscle) are toxic when given intravenously to rabbits, he does not give a single protocol in which liver extract was injected. Schenk,[7] however, gives one experiment which demonstrates the toxicity of nor-

[1] Unpublished.

[2] Manwaring, W. H., *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 589.

[3] Weil, R., *J. Immunol.*, 1917, ii, 525.

[4] Dold, H., *Z. Immunitätsforsch., Orig.*, 1911, x, 53.

[5] Loeb, L., *Z. Immunitätsforsch., Orig.*, 1912, xii, 189.

[6] Dold, H., and Ogata, S., *Z. Immunitätsforsch., Orig.*, 1912, xiii, 667.

[7] Schenk, F., *Z. Immunitätsforsch., Orig.*, 1914, xxii, 229.

mal liver, but does not say how his extract was prepared and he may have used very large doses.

An experiment on normal tissue extracts is given below which confirms Dold's work except in the case of the liver.

EXPERIMENTAL.

A normal rabbit was bled to death under ether. Pieces of the organs noted (Table I) were placed in sterile test-tubes, weighed, ground in a sterile mortar with sand, and emulsified in salt solution added in the proportion of 5 cc. to 1 gm. of tissue. The mixture was shaken in large sterile tubes in the machine for 10 minutes and centrifugalized at high speed for half an hour. The slightly turbid supernatant fluid was pipetted off and injected into rabbits. This technique was used in all the following experiments and all inoculations were made intravenously.

TABLE I.

Rabbit No.	Weight.	Amount injected.	Organ extract.	Remarks.
	gm.	*cc.*		
1	1,840	10	Liver.	No symptoms.
2	1,900	5	Lung.	Dead in 1 min.
3	1,605	5	Kidney.	" " 1 "
4	1,250	5	Brain.	" " 2 "

We have made many experiments with normal liver extracts giving 10 cc. to rabbits weighing over 1,000 gm. and 5 cc. to those below this weight, and in only one instance was a reaction obtained. This was in a rabbit weighing 1,830 gm. which had been injected with 10 cc. of liver extract. Rapid breathing and prostration developed 2 minutes after the injection; recovery was complete in 20 minutes. A second rabbit, weighing 1,730 gm., received 10 cc. of extract of the same liver without reaction.

At the outset various doses of living typhoid bacilli (Rawling's strain) were injected intravenously into rabbits which were bled to death under ether when very sick. A piece of liver was then removed and extracted as described above. It was later found that the liver became highly toxic when even boiled typhoid antigen was

injected. However, most of the experiments were made with living typhoid bacilli. Two protocols illustrating the latter are given.

Experiment 1.—Rabbit 5; weight 1,250 gm. Injected with 1 agar slant of typhoid bacilli. Death occurred 5 hours later. Liver extracted as usual.

Rabbit 6; weight 1,050 gm. Injected with 5 cc. of the liver extract of Rabbit 5. Convulsion in 1 minute; death in 1½ minutes.

Experiment 2.—Rabbit 7; weight 1,290 gm. Injected intravenously with ⅔ agar slant of typhoid bacilli. No immediate effect, but 2 hours later the animal was weak, and during the day the weakness increased; bled and killed while in collapsed condition 8 hours after the inoculation. Liver removed and extracted as indicated.

Rabbit 8; weight 1,185 gm. Given intravenously 10 cc. of the extract. Immediately on conclusion of the injection the animal became prostrated, then rose, ran a short distance, and died; survived injection 1 minute.

By further experimentation it was found that in order to obtain rabbits in which the liver was definitely toxic the dose of typhoid bacilli had to be so adjusted that the animal was rendered very sick 4 to 8 hours after the injection, at which time it must be killed. It is difficult to make a definite rule as to what this dose should be, for rabbits vary widely in their resistance to typhoid bacilli. If an overwhelming injection is given (2 to 4 slants) so that the rabbit succumbs in about 2 hours, the liver is non-toxic. In order that a liver may be rendered sufficiently toxic, time must be allowed it, not only to take up the bacteria, kill, and disintegrate them, but also to elaborate the poisonous products. With the strain (Rawling's) used in this study the most constant results were obtained by injecting 1 agar slant of the bacilli.

Experiments were made with typhoid antigen which was prepared as follows: The 48 hour growth from 40 potato tubes of agar was washed off with isotonic salt solution and poured into centrifuge tubes, and the suspensions were centrifugalized at high speed. After pipetting off the clear supernatant fluid, the sediment was taken up in 100 cc. of salt solution, 0.2 N sodium hydroxide added to it, and the whole heated to 58°C. for ½ hour, and then shaken several hours a day for 5 days. Microscopic examination of this material which constituted the antigen showed the bacteria to be well broken up and staining very feebly.

In respect to the employment of the antigen for rendering the liver toxic, it is even more difficult to indicate the effective dose. On its inoculation the animal ordinarily falls sick soon after the injection and either gets progressively worse till death occurs or the condition improves after a couple of hours. In the latter instance a second injection must be given. The liver becomes toxic in a shorter time after antigen administration than after injection of the bacilli.

The following protocols are those of an experiment in which the antigen was given.

Experiment 3. One Dose of Antigen.—Rabbit 9; weight 1,230 gm. Injected with 0.5 cc. of the typhoid antigen. After 1 hour, diarrhea, and prostration which progressed. Killed 5½ hours after inoculation.

Liver extract was prepared from this rabbit as before. 10 cc. were injected into Rabbit 10, weighing 1,440 gm. There were immediate convulsion and death in 1½ minutes.

Experiment 4. Two Doses of Antigen.—Rabbit 11; weight 1,560 gm. Given 1 cc. of the antigen. 2½ hours later very sick. During next 2 hours partial recovery. Injected with 2 cc. of the antigen. Bled and killed 8 hours after first injection. The liver extract was prepared, and 5 cc. were injected into Rabbit 12, weighing 950 gm. Immediate convulsion; death in 3 minutes.

Experiment 5. Two Doses of Antigen.—Rabbit 13; weight 1,275 gm. Given 0.5 cc. and 1½ hours later given 2 cc. of the antigen. Killed 5 hours after injection. 10 cc. of the liver extract were injected into Rabbit 14, weighing 1,945 gm. Immediate convulsion and death in 3 minutes.

The boiled antigen is active but less so apparently than the unboiled. Two tests only were made with it; in neither did the liver extract kill the second animal, possibly because the first rabbits were not killed at the proper time.

A protocol in which the boiled antigen was used is given, as it illustrates the symptoms which arise from a sublethal dose of the liver poison.

Experiment 6.—Rabbit 15; weight 1,330 gm. Given 1.5 cc. of the typhoid antigen which had been boiled for 5 minutes. The animal became sick in less than 1 hour and remained so till killed 5 hours later. 10 cc. of the liver extract were injected into Rabbit 16, weighing 1,230 gm. 2 minutes later breathing was labored and slow, the head and abdomen resting on the floor. 16 minutes after injection it jumped up and ran a short distance, but fell again from weakness. After 30 minutes, breathing better, and animal stronger. 1½ hours after the injection, appeared almost normal. Well next morning.

10 cc. of a Berkefeld filtrate of the same liver extract were injected into Rabbit 17, weighing 1,340 gm. After 3 minutes labored breathing and weak. After 10 minutes flattened out on table; breathing very labored. After ½ hour recovering; well next morning. This experiment will be referred to again.

The toxic liver tissue changes at ice box temperature and after 2 days it is no longer poisonous. The next experiment indicates this fact and also shows that the normally toxic lung is not detoxicated under similar conditions.

Experiment 7.—Rabbit 18; weight 1,810 gm. Given 0.5 cc. of the typhoid antigen. Death occurred 65 minutes later. One piece of the liver and one piece of the lung were prepared as usual. A second piece of the liver and of the lung was placed in the ice box for 36 hours. 5 cc. of the usual liver extract from the liver of Rabbit 18 were inoculated into Rabbit 19, weighing 950 gm. In 1 minute convulsion, in 2 minutes death. 5 cc. of the liver extract prepared from the piece of liver which had been on ice were injected into Rabbit 20, weighing 930 gm.; no effect produced. 3 cc. of an extract from the lung which had been on ice were injected into Rabbit 21, weighing 1,540 gm. Dead in 1½ minutes. This test with liver was repeated with the same results. A sufficient number of experiments has not been done with the ordinary toxic liver extracts to know how long the extracted poisons will keep in the ice box. However, the fact that it has not deteriorated much in 2 days has been determined several times.

It seems probable that this detoxicating power of the liver is responsible for the difficulty in obtaining poisonous extracts. Probably the normal liver is able to neutralize, as they are formed, large amounts of poison, and it is only when the quantity produced exceeds the detoxicating power of the liver that the poison can be demonstrated. Possibly poisons are also formed from bacteria in other organs, but this point is difficult to prove on account of the toxicity of the normal organs. The fact that in rabbits the liver takes up from forty to fifty times as many bacteria as all the other organs together[1] argues in favor of its being the principal seat of the poison formation. It is interesting to note here that Weiss, Kolmer, and Steinfield[8] have recently noted that pneumonic lungs from man and dog are more toxic to laboratory animals than normal lungs.

Dold,[4] Dold and Ogata,[6] and Loeb[5] found that normal organs caused death because of a coagulative ferment, which leads to the

[8] Weiss, C., Kolmer, J. A., and Steinfield, E., *J. Infect. Dis.*, 1918, xxii, 469.

formation of thrombi in the right heart and pulmonary artery and veins. We have corroborated these findings. These writers also found that the ferment does not pass through a Berkefeld filter, and that it is destroyed by heating to 60°C. for 1 hour.

We have searched many times for thrombi in the right heart and pulmonary artery in rabbits that have succumbed to the liver poison, but in only four out of twenty-six animals autopsied immediately after death was a small clot found in the right heart. In one animal there was a clot also in the pulmonary artery. In the animals that succumbed to the liver poison the heart beats several minutes after the cessation of respiration, and the blood coagulates slowly.

Several tests were made with a Berkefeld filtrate of the typhoid livers, and although the filtrate was less toxic than the original extract, it was still poisonous as shown in Experiment 6, Rabbit 17.

Boiling of the toxic extract was active in one of the three tests.

Experiment 8.—10 cc. of the liver extract used in Experiment 4 were boiled for 5 minutes. The coagulum was centrifugalized off and 6.5 cc. of the supernatant fluid were injected into a rabbit, weighing 1,280 gm. Within 2 minutes the animal was prostrate, and there was "anaphylactic" breathing. In 5 minutes the symptoms abated and in 10 minutes they had passed off.

To exclude the blood as the source of the toxicity, 3.4 cc. of serum and 4 cc. of defibrinated blood taken from animals whose liver extract was toxic, were injected into two rabbits, weighing 600 gm. each. No symptoms resulted.

Experiments with Bacillus prodigiosus.

The next series of tests was made with another bacterial species in order to test the specificity of the poison produced. *Bacillus prodigiosus* was selected because of its toxicity for rabbits.

After many fruitless efforts it was found that a toxic liver extract could be obtained with *Bacillus prodigiosus* provided the injected animal was killed very soon after inoculation. Our best results were obtained from 2 or 3 slants of *Bacillus prodigiosus* and by killing the rabbit while still prostrate and from 1 to 3 hours after the injection. The effects are quite the same whether the toxic liver extract is made with typhoid or *prodigiosus* bacilli.

Experiment 9.—A normal rabbit weighing 1,665 gm. was given 2 agar slants of *B. prodigiosus;* killed while sick 1 hour and 10 minutes later. With the extract of the liver of this animal three rabbits were injected (Table II).

TABLE II.

Rabbit No.	Weight.	Amount injected.	Remarks.
	gm.	*cc.*	
22	1,610	5	Dead in ½ min.
23	1,440	3	" " 4 "
24	1,240	2	Very sick. Died during night.

Experiment 10.—Rabbit 25; weight 1,420 gm. Given 3 agar slants of *B. prodigiosus.* Killed 3¾ hours later. 5 cc. of the liver extract were injected into another rabbit, weighing 1,365 gm. Death was immediate.

Experiments on Immunity to the Liver Poison.

The question as to whether rabbits could be immunized to the liver poison was investigated. Although testing of resistance to multiple lethal doses of the poison was made difficult by reason of the large quantities of extract to be injected, yet it developed that in many instances one or more injections of the poison gave rise to tolerance to 1½ lethal doses persisting at least 10 days. In this respect also the liver poison differs from that found in extracts of normal organs by Dold and Ogata.[6] These writers also produced tolerance to the normal organ poison, but it lasted only 24 to 48 hours.

Two examples of the phenomenon mentioned follow:

Experiment 11.—Rabbit 26 was immunized to *prodigiosus* liver poison as follows:
Jan. 12, 1918. Weight 1,480 gm. 5 cc. of the liver extract (sublethal dose).
Jan. 18. Weight 1,470 gm. 7 cc. of the liver extract (1⅓ lethal doses).
Jan. 23. Weight 1,690 gm. 8 cc. of the liver extract (1½ lethal doses).
Jan. 28. Weight 1,580 gm. 8 cc. of the liver extract (1⅓ lethal doses).
Rabbit 27 was immunized to typhoid liver poison as follows:
Jan. 8, 1918. Weight 2,440 gm. 10 cc. of the liver extract (½ lethal dose).
Jan. 15. Weight 2,350 gm. 7 cc. of the liver extract (¾ lethal dose).
Jan. 19. Weight 2,500 gm. 10 cc. of the liver extract (1 lethal dose).
Jan. 23. Weight 2,480 gm. 12 cc. of the liver extract (1⅓ lethal doses).
Feb. 7. Weight 2,525 gm. 12 cc. of the liver extract (1½ lethal doses).

Moreover, it was found that rabbits actively immunized to *Bacillus typhosus* or *Bacillus prodigiosus* resist respectively the typhoid and *prodigiosus* liver extracts.

Experiment 12.—Rabbit 28, weighing 2,250 gm., was injected with 1 agar slant of *B. typhosus*; killed after 7 hours when very sick. An actively immune rabbit, weighing 2,200 gm., was given 10 cc. of the liver extract from Rabbit 28. No symptoms appeared. A normal rabbit, weighing 2,250 gm., was given 10 cc. of the same liver extract. Dead in 8 minutes after injection. The history of the actively immune rabbit is as follows:

Dec. 3, 1917. Weight 2,265 gm. $\frac{1}{10}$ culture of live typhoid bacilli (Rawling's).
Dec. 10. Weight 2,035 gm. $\frac{1}{5}$ culture of live typhoid bacilli (Rawling's).
Dec. 15. Weight 2,220 gm. $\frac{1}{2}$ culture of live typhoid bacilli (Rawling's).
Dec. 22. Weight 2,210 gm. $\frac{3}{4}$ culture of live typhoid bacilli (Rawling's).
Dec. 29. Weight 2,230 gm. 1 slant culture of live typhoid bacilli (Rawling's).
Jan. 7, 1918. Weight 2,200 gm. Injected with the liver extract.

The next step was to determine whether typhoid immune serum was capable of neutralizing or destroying the typhoid liver poison *in vitro* and *in vivo*. For this purpose a lethal dose (10 cc.) of the typhoid liver poison was mixed with 2 cc. of high titer typhoid immune serum and kept at 37°C. for 1 hour. The controls consisted of normal serum and salt solution instead of the immune serum. It developed that even the salt solution control became detoxicated by this treatment. The experiment was repeated twice, the procedure being the same except that in one instance the mixtures were kept at 23°C. and in the other at 0°C. for 1 hour. No differences were noted.

No explanation is offered at present to account for the destruction of the toxic extract on mere standing with saline solution. A modification of the method of procedure also failed to bring out neutralizing power of the immune serum. Thus three pieces of the liver taken from a rabbit dying of typhoid liver extract were treated respectively (*a*) with isotonic salt solution as usual, (*b*) extracted with 1:20 normal, and (*c*) 1:20 typhoid immune serum. The three mixtures were kept at 0°C. for 2 hours before injection. Upon inoculation it was found that the immune serum liver extract killed a rabbit in even less time than the salt solution control. The immune serum exerted, therefore, no detoxicating effect.

The *in vivo* test was made as follows: Rabbits 29 and 30 were injected each with 3 cc. of immune (1:40,000 titer) and of normal serum respectively and 2 minutes later were given a lethal dose of the liver poison. Both animals survived. This experiment was repeated several times with the same result. Then two other rabbits were given the normal and immune rabbit sera, $5\frac{1}{2}$ hours before the injection of the toxic liver extract. Both these died. Here again no explanation is offered for this phenomenon which calls for minute study and analysis.

As far as the tests described go it may be stated that typhoid immune serum does not protect specifically against the liver poison.

Specificity of the Poisons.

The opinion first entertained was that probably the liver poisons were related closely to those discovered by Friedberger and Nathan[9] or to the protein split products of Vaughan,[10] but the experiments on their specificity caused a modification of that view. Two sets of tests bearing on this point were made. In the first, rabbits were rendered refractory to the typhoid and *prodigiosus* poisons respectively and then tested against the heterologous poison as follows:

Experiment 13.—Rabbit 31, weight 1,560 gm., was given 10 cc. of a typhoid liver extract and died in 1 minute. Rabbit 32, weight 2,515 gm., had been given previously six graded doses of the typhoid liver poison. It was then given 12 cc. of the typhoid liver poison without effect. Rabbit 33, weight 1,650 gm., had been given four graded doses of the *prodigiosus* liver poison. It was then given 8 cc. of the typhoid liver poison. Death in 1 minute.

The experiment was modified and carried out in immune typhoid and *prodigiosus* animals.

Experiment 14.—A normal rabbit was inoculated with 1 agar slant of *B. typhosus* and killed when dying 4 hours later. The liver extract was inoculated into other rabbits (Table III).

The immune rabbits used in these experiments were prepared as shown in Table IV.

[9] Friedberger, E., and Nathan, E., *Z. Immunitätsforsch., Orig.*, 1911, ix, 444.

[10] Vaughan, V. C., Jr., and Vaughan, J. W., Protein split products in relation to immunity and disease, Philadelphia and New York, 1913.

It should be stated that a 24 hour agar slant of *Bacillus prodigiosus* contains at least twice as many bacteria as one of *Bacillus typhosus*.

The experiment was now revised so that the *prodigiosus* liver poison was injected into immune typhoid and *prodigiosus* rabbits.

Experiment 15.—A rabbit weighing 1,220 gm. was injected with 2 slants of *B. prodigiosus* and killed 2½ hours later when very sick. The liver extract was inoculated into other rabbits (Table V).

TABLE III.

Rabbit No.	Condition.	Weight.	Amount injected.	Remarks.
		gm.	*cc.*	
34	Normal.	1,350	8	Dead in 2 min
35	Typhoid-immune.	1,345	8	No symptoms.
36	"	1,645	8	" "
37	"	1,850	10	Slightly sick.
38	"	1,900	10	Convulsion in 4 min. Dead in 9 min.
39	*Prodigiosus*-immune.	1,555	8	" " ½ " " " 2½ "
40	"	2,280	10	" " 1½ " " " 4 "

TABLE IV.

Rabbit No.	Organism injected.	Amount injected.	No. of injections.	Agglutination titer of serum for *B. typhosus*.
35	Typhoid.	1.2 slants.	7	1:4,000
36	"	0.7 slant.	6	1:20,000
37	"	0.9 "	7	1:40,000
38	"	0.8 "	6	1:4,000
39	*Prodigiosus*.	0.4 "	8	None at 1:100.
40	"	0.3 "	7	" " 1:100.

TABLE V.

Rabbit No.	Condition.	Weight.	Amount injected.	Remarks.
		gm.	*cc.*	
41	Normal.	1,600	8	Convulsion in 1 min. Dead in 2 min.
42	Typhoid-immune.	1,790	7	Very sick. Convulsion in 5 min. Dead in 7½ min.
43	"	1,740	7	Sick. Convulsion. Dead in 45 min.
44	*Prodigiosus*-immune.	1,540	7	Very sick for 3 min. Recovered in 4 min.
45	"	1,540	7	Sick for 8 min. and then recovered.

The immune rabbits used in the experiment were prepared as shown in Table VI.

TABLE VI.

Rabbit No.	Organism injected.	Amount injected.	No. of injections.
42	Typhoid.	1.7 slants.	12
43	"	1.0 slant.	8
44	*Prodigiosus.*	0.5 "	8
45	"	0.4 "	8

Experiment 16.—In this experiment the typhoid liver extract was prepared from a rabbit, weighing 1,350 gm., which had been given 1 slant of *B. typhosus* and was killed when moribund 4 hours later. The *prodigiosus* liver extract was prepared from a rabbit, weighing 1,700 gm., which had been given 2 slants of *B. prodigiosus* and killed when very sick 1½ hours later.

The results of the experiment are shown in Table VII.

TABLE VII.

Rabbit No.	Condition.	Weight.	Amount injected.	Toxic liver extract.	Remarks.
		gm.	*cc.*		
46	Normal.	1,720	8	*Prodigiosus.*	Dead in 2 min.
47	Typhoid-immune.	1,830	8	"	" " 4½ "
48	*Prodigiosus*-immune.	1,720	8	"	Severe symptoms. Recovered.
49	Normal.	1,560	10	Typhoid.	Dead in 1 min.
50	*Prodigiosus*-immune.	1,840	10	"	" " 1½ "
51	Typhoid-immune.	1,985	10	"	Slight symptoms.

The immune rabbits of this experiment had been given at least five injections of either *Bacillus typhosus* or *Bacillus prodigiosus*.

The results of the experiment with actively immune animals indicate, therefore, that the liver poisons employed in them are specific. This point is an interesting one in view of the fact that specific immune sera for *Bacillus typhosus* and *Bacillus prodigiosus* do not neutralize the poisons. The explanation of this discrepancy is not at once apparent and further study is required to elucidate the phenomenon.

Possibly it is bound up with cellular in contradistinction to serum immunity.

DISCUSSION.

The experiments described in this paper make it probable that part at least of the intoxication produced in rabbits by injections of *Bacillus typhosus* or *Bacillus prodigiosus* is due to liver poisons of the nature of those dealt with here and which appear to be yielded to the blood by the liver, and then absorbed by the various tissues. It should not be surprising, perhaps, that the poisons in the quantities used in these experiments should be insufficient in amount to be demonstrable in the blood, for they are probably being removed from the blood continuously and with considerable speed by the tissues. Hence, only very small quantities are probably present at any one time in the circulation even when the animal is extremely sick.

Rabbits actively immune to *Bacillus typhosus* or *Bacillus prodigiosus* exhibit a condition of resistance apparently because of having acquired in the process of immunization a tolerance to the liver poison. For it is possible that even if several lethal doses of the bacteria are injected into the immune animal, not more than one lethal dose of the liver poison is produced in a unit of time, an amount which the immune animal can tolerate readily.

Probably the liver poisons are of cellular origin and are produced by the living liver cell and possibly by the cells of other organs also acting upon the typhoid or *prodigiosus* bacteria, or their disintegrated products, which fact explains why they are not produced outside the body of the animal.

Whether a similar poison is generated in typhoid fever in man is a question that cannot now be answered. It seems not impossible that it may play a part in causing the symptoms of toxemia in that disease.

CONCLUSIONS.

1. The livers of rabbits inoculated with cultures of *Bacillus typhosus* or *Bacillus prodigiosus* under certain conditions contain a toxic substance extractable with salt solution. When the toxic extracts are injected intravenously into normal rabbits the latter animals develop

symptoms resembling those of anaphylactic shock and succumb. The lethal doses of the toxic extracts are far smaller than those of normal liver extract.

2. The livers of rabbits injected with typhoid antigen also yield a toxic extract.

3. Boiling as well as filtration through a Berkefeld filter only partially detoxicates the extract.

4. Tolerance to one to two lethal doses of the poisonous extracts can be induced by cautious immunization.

5. Rabbits actively immunized to *Bacillus typhosus* or *Bacillus prodigiosus* usually resist one lethal dose of the homologous liver poison; and animals tolerant to the typhoid liver poison resist one minimum lethal dose at least of *Bacillus typhosus*.

6. Typhoid immune serum is not detoxicating either *in vivo* or *in vitro* for the typhoid liver poison.

7. The liver poisons are specific, since rabbits actively immunized to either *Bacillus typhosus* or *Bacillus prodigiosus* withstand at least one minimum lethal dose of the homologous but not of the heterologous liver poisons.

It is a pleasure to acknowledge the invaluable services of Mr. James May, whose skilled assistance contributed essentially to the success of the experiments.

AN IMPROVEMENT IN THE METHOD OF ISOLATING AND RECOVERING THE BACILLUS OF CATTLE ABORTION THROUGH GUINEA PIGS.

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History of the Test.

For the isolation of *B. abortus* Bang up to 1912 there were available the culture methods of Bang and Nowak. Early in 1912 Theobald Smith and Fabyan[1] showed that *B. abortus* inoculated into guinea pigs produces a disease with well defined characters, and that the disease can be isolated from these guinea pigs after 3 or more months. This method proved a valuable addition inasmuch as culture methods frequently failed when badly soiled fetal membranes were objects of investigation.

This inoculation disease had already been recognized as an entity, distinct from tuberculosis, in 1894 by Smith,[2] and Schroeder and Cotton[3] had the disease under observation since 1911 ascribing it, however, to a Gram-positive bacterium. This error was rectified in a publication issued in March, 1912.[4] The disease in guinea pigs was more fully described by Fabyan[5] in 1912. Since that date the guinea pig has been used by various workers for the isolation of *B. abortus* from fetal membranes and fetal tissues.

In view of the value of the method it seemed desirable to determine whether it might be possible to shorten the life period of the inoculated guinea pig without impairing the chances for obtaining cultures. In the following pages a number of experiments are described which were planned with this object in view.

[1] Smith, T., and Fabyan, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1912, lxi, 549.

[2] Schroeder, E. C., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 7*, 1894, note by T. Smith, p. 80.

[3] Schroeder, E. C., and Cotton, W. E., *Proc. Am. Vet. Med. Assn., 48th Convention*, 1911, 442.

[4] *U. S. Dept. Agric., Bureau Animal Industry, Circular 198*, 1912.

[5] Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.

EXPERIMENTAL.

The material used to inoculate guinea pigs was derived from pure cultures as well as from tissues of fetuses and fetal membranes.

The guinea pigs were inoculated either into the subcutis or the abdominal cavity. When the animals were considered ready for bacteriological examination they were chloroformed, autopsied, the lesions noted, and the following procedure, which is the one regularly used in this laboratory for the cultivation of *Bacillus abortus*, was adopted throughout.

Ordinary veal peptone agar tubed and slanted is the medium employed. The surface of the organs from which cultures are to be made is, if necessary, seared with a heated spatula. Bits of tissue about the size of split peas are then torn out of the organ with sterile forceps, rubbed over the entire surface of the agar with a platinum loop, and finally pushed down into the condensation water. The cotton plugs are clipped off level with the tops of the tubes. The tubes at this point are thoroughly heated in the flame to kill any spores adhering to the contained cotton plugs as a result of handling, and after the tubes have cooled somewhat they are hermetically sealed with a layer of sealing wax. They are then incubated at 37°C. Colonies of *Bacillus abortus* are usually observed on the agar slant after 5 to 10 days incubation.

Cultures were prepared from lungs, liver, kidneys, and spleen in all cases; from the superficial inguinal lymph nodes when the animal had received a subcutaneous injection; from the retrogastric lymph nodes when the injection was intraabdominal; and from the testicle and the ovary or uterus. After an incubation of from 6 to 10 days, the cultures were examined and any growth was noted and studied. The colonies were counted on the agar surface. In case no growth appeared, the condensation water containing the bit of tissue was shaken so as to cover the agar surface and the tubes were replaced in the incubator. The object of this procedure was to give any bacteria growing out of the bit of tissue or in the condensation water an opportunity to multiply on the inclined surface. Probably one out of every ten to twenty tubes responded to this treatment.

To identify *Bacillus abortus* the colonies were examined both macroscopically and microscopically. The macroscopic characteristics of

such colonies are in themselves almost diagnostic. They vary considerably in size according to the numbers on the agar surface. When crowded they are relatively minute and as a rule discrete, except near the margin of the condensation water. When 1 to 2 cm. apart, they may become 3 mm. in diameter and strikingly convex. The microscopic examinations of these colonies were made in all instances to verify the macroscopic inspection, and the Gram test was applied to differentiate *Bacillus abortus* from Gram-positive organisms, such as *Bacillus pyogenes*.

The diagnosis of infection with *Bacillus abortus* was furthermore strengthened by a macroscopic examination of the organs of every guinea pig. As is now well known, in positive cases the spleen is much enlarged, highly congested, more or less nodular on the surface, and the Malpighian bodies may be enlarged and show through the distended capsule and on section as grayish foci of varying sizes. In addition to this characteristic lesion, one or both testicles may be attacked and the epididymis converted into an indurated, centrally necrotic mass. Minute nodules in the liver are found in most cases, usually not quite a millimeter in diameter, sometimes yellowish and probably representing necroses, sometimes grayish or pearly and then representing minute collections of cells as described by Smith and Fabyan and Fabyan.

Series I.

The first series of guinea pigs injected consisted of twelve, six males and six females, varying between 300 and 400 gm. in weight. They were injected September 26, 1917, with a stock culture recovered from a fetus after passage through a guinea pig. Three of each sex were inoculated subcutaneously and the same number intraperitoneally, each individual receiving 1 cc. of a suspension of the strain. The suspension was prepared by washing off the surface colonies of a 72 hour agar slant with sterile salt solution, diluting the resulting suspension to the approximate density of a 24 hour bouillon culture of *Bacillus typhosus* and then again diluting fifty times. Table I summarizes the results. The enlargement of the spleen is designated in bulk and not in dimensions.

TABLE I.

Results of Inoculation of Guinea Pigs with a Pure Culture of B. abortus Isolated through Guinea Pigs from a Fetus.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + / Loss in weight −	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
1	Male.	Intraperitoneal.	15	+ 6	Six times normal size; adherent to ribs.	—	Spleen: luxuriant growth. Liver: good " Testicle: " "	+
2	Female.	Subcutaneous.	15	+14	Five times normal size; hyperemic and friable.	—	Spleen: " " Regional lymph node: good growth. Kidney: good growth. Liver: few colonies.	+
3	Male.	Intraperitoneal.	21	+22	Eight times normal size; easily torn; surface nodular; nodules numerous.	Small necrotic foci in liver.	Spleen: 200 Regional lymph node: 150 Testicle: 18 Lung (contaminated): 18 Kidney: 2	+
4	Female.	Subcutaneous.	21	+13	Eight times normal size, surface nodular.	Small abscess at point of injection.	Spleen: 100 Regional lymph node: 200 Lung: 31 Kidney: 6 Ovary: 1	+

5	Male.	Intraperitoneal.	29	+20	Slightly enlarged; very dark; surface nodular.	Minute necrotic foci in liver.	Spleen:	150	+
							Retrogastric lymph node:	50	
							Kidney:	7	
							Lung:	3	
							Liver:	2	
6	Female.	Subcutaneous.	29	+52	Six times normal size; surface nodular.	Numerous minute necrotic foci in liver.	Spleen:	150	+
							Regional lymph node:	125	
							Lung:	4	
							Ovary:	3	
							Liver:	2	
7	"	Intraperitoneal.	35	−15	Eight times normal size; adherent to peritoneum and left kidney; tubercle in spleen at adherent point.	Few minute necrotic foci in liver. Capsule of left kidney thickened.	Spleen:	300	+
							Retrogastric lymph node:	300	
							Kidney:	300	
							Lung:	6	
							Liver:	3	
							Ovary:	2	
8	Male.	Subcutaneous.	35	+87	Markedly enlarged; easily torn; rounded borders.	Liver enlarged; minute scattering tubercles.	Spleen:	14	+
							Regional lymph node:	140	
							Lung:	9	
							Testicle:	6	
							Kidney:	5	
							Liver:	2	
9	Female.	Intraperitoneal.	43	−29	Nine times normal size; surface smooth and easily torn.	—	Spleen:	100	+
							Retrogastric lymph node:	5	
							Kidney:	8	
							Lung:	5	
							Liver:	2	
							Uterus:	2	

TABLE I—*Concluded.*

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + / Loss in weight −	Condition of spleen.	Other lesions.	No. of colonies on agar slant.		Result.
			days	*gm.*					
10	Male.	Subcutaneous.	42	+90	Twice normal size; hyperemic and surface nodular.	One tubercle in liver 3 mm. in diameter.	Spleen:	80	+
							Regional lymph node:	60	
							Liver:	3	
							Kidney:	3	
							Testicle:	2	
							Lung:	0	
11	Female.	Intraperitoneal.	52	−35	Enormously enlarged; adherent to peritoneum and left kidney; nodular surface.	Minute scattering tubercles and small necrotic foci in liver.	Spleen:	75	+
							Retrogastric lymph node:	50	
							Kidney:	12	
							Lung:	7	
							Uterus:	4	
							Liver:	0	
12	Male.	Subcutaneous.	52	+106	Enormously enlarged; nodular surface; small glistening tubercles.	Minute irregular necrotic areas in liver.	Spleen:	75	+
							Regional lymph nodes:	150	
							Kidney:	20	
							Liver:	3	
							Lung:	3	
							Testicle:	0	

Series II.

Six female guinea pigs, weighing between 325 and 375 gm. each, were used in the second series. They were inoculated September 26, 1917, three subcutaneously and three intraabdominally with a culture of *Bacillus abortus*, recovered from the placenta of a cow, after passage through a guinea pig. The method of preparation of the material to be injected and the dosage were the same as for the guinea pigs of Series I. A condensed description of Series II is given in Table II.

Series I and II were inoculated with the strains from a certain fetus and a certain placenta from two herds respectively, to determine whether or not there was an appreciable difference in the pathogenicity. It will be noted from the tables that the extent of the lesions produced, and the comparative colony counts for the two strains are approximately the same. As far as the guinea pig test is concerned the strains are evidently of the same level of virulence.

Series III.

The third series of guinea pigs consisted of six males and six females weighing between 350 and 450 gm. each. Three guinea pigs of each sex were inoculated subcutaneously, and the same number of each sex intraabdominally, on October 24, 1917, with a salt solution suspension of crushed cotyledons from the placenta of another cow.

The material to be injected was prepared in the following manner: Five cotyledons that were apparently affected were removed into sterile Petri dishes. After they had been thoroughly washed in running tap water from a deep well to remove particles of straw, etc., pieces varying in size from 1 to 2 gm. were clipped from each cotyledon, care being taken to cut down to the base of the villi. The bits of tissue were then ground up with sterile quartz sand in a sterile mortar. The ground mass was suspended in 0.85 per cent salt solution until a density was reached which compared with a 24 hour bouillon culture of *Bacillus typhosus*. Each animal was injected with 1 cc. of this suspension. Table III is a summary of the results.

An analysis of the three foregoing experiments indicates that the inoculation disease in guinea pigs due to *Bacillus abortus* runs a fairly definite course. The number of colonies appearing in cultures is

TABLE II.

Results of Inoculation of Guinea Pigs with a Pure Culture of B. abortus Isolated through Guinea Pigs from the Placenta of a Cow.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + / Loss in weight −	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
13	Female.	Intraabdominal.	16	−26	Twice normal size; congested; easily torn.	—	Spleen: luxuriant growth. Retrogastric lymph node: luxuriant growth. Kidney: few colonies. Liver: " " Lung: " "	+
14	"	Subcutaneous.	16	+16	Normal in size and appearance.	Minute grayish necrotic foci in liver. Small abscess (8 mm. diameter) at site of injection.	Spleen: 200 Liver: 20 Kidney: 15 Lung: 3	+
15	"	Intraperitoneal.	28	+21	Five times normal size; surface nodular.	Scattering grayish (necrotic) foci in liver.	Spleen: 200 Retrogastric lymph node: 48 Liver: 10 Kidney: thin film. Lung: " "	+

16	Female.	Subcutaneous.	28	+58	Four times normal size; nodular surface.	Minute necrotic areas in liver. Small subcutaneous abscess at site of injection.	Spleen: Regional lymph node: Lung: Liver: Kidney: Ovary: thin film.	175 300 8 4 3	+
17	"	Intraperitoneal.	50	+41	Eight times normal size; congested; surface smooth and easily torn.	Minute (necrotic) areas in liver; walls of uterus injected.	Spleen: Retrogastric lymph node: Kidney: Liver: Lung: Uterus: few.	100 40 4 2 1	+
18	"	Subcutaneous.	50	+116	Four times normal size; marked congestion; small tubercles; nodular surface.	A few scattering nodules in liver.	Spleen: Regional lymph node: Kidney: Liver:	125 10 6 4	+

TABLE III.

Results of Inoculation of Guinea Pigs with Salt Solution Suspensions of Placenta from a Case of Abortion.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days.	*gm.*				
19	Female.	Subcutaneous.	15	+15	Twice normal size; congested; easily torn.	—	Spleen: 60 Regional lymph node: 200 Kidney: 20 Lung: 1 Liver: 1	+
20	Male.	Intraperitoneal.	16	− 9	Slightly enlarged; surface slightly nodular.	—	Spleen: 8 Retrogastric lymph node: 5 Lung: 8 Liver: 2 Kidney: 1 Testicle: 0	+
21	"	Subcutaneous.	22	+17	Slightly enlarged; surface nodular.	Minute, scattering, refractile tubercles in liver.	Spleen: 150 Regional lymph node: countless. Kidney: 90 Lung: 8 Testicle: 1 Liver: 1	+

22	Female.	Intraperitoneal.	22	+53	Slightly swollen; congestion; surface nodular.	A few minute scattering tubercles.	Spleen: countless. Retrogastric lymph node: 70 Kidney: 100 Liver: 5 Lung: 5 Uterus: 0	+
23	"	"	44	+153	Somewhat enlarged; congested; nodular surface.	Minute grayish necrotic foci in liver; few small tubercles.	Spleen: 35 Retrogastric lymph nodes: 10 Lung: 2 Uterus: 1 Kidney: 1 Liver: 0	+
24	Male.	Subcutaneous.	44	+125	Twice normal size; congested and very dark; nodular surface.	Fibrous induration of right testicle; disintegration of epididymis.	Spleen: 70 Regional lymph nodes: 10 Testicle: 1 Liver: 1 Lung: contaminated.	+
25	"	Intraperitoneal.	49	+151	Twice normal size; congested; prominent nodules on surface.	Minute necrotic foci in liver; large colon adherent in sublumbar region; abscess at adherent point.	Spleen: 30 Liver: 4 Lung: 1 Kidney: 1 Testicle: 1	+
26	Female.	Subcutaneous.	49	+98	Six times normal size; congested; smooth, easily torn surface.	Minute yellowish gray necrotic foci in liver.	Spleen: 45 Regional lymph node: 35 Liver: 1 Kidney: 1 Lung: contaminated.	+

TABLE III—*Concluded.*

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + / Loss in weight −	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
27	Female.	Intraperitoneal.	28	+47	Twice normal size; surface nodular.	A few minute tubercles in liver.	Spleen: 250 Retrogastric lymph node: 80 Liver: 23 Uterus: 1 Kidney: many. Lung: contaminated.	+
28	Male.	Subcutaneous.	29	+11	Slightly enlarged; congested; surface nodular.	Minute necrotic foci in liver; a few scattering refractile tubercles; subcutaneous abscess at site of injection.	Spleen: 150 Regional lymph node: 125 Liver: 2 Kidney: 2 Lung: contaminated.	+
29	"	Intraperitoneal.	38	+99	Slightly enlarged; congested; nodular surface; fibrosis of the center.	Omentum adherent to peritoneum in two places.	Spleen: 90 Retrogastric lymph node: 75 Kidney: 4 Lung: 1 Liver: 0 Testicle: 0	+
30	Female.	Subcutaneous.	38	+125	Eight times normal size; congested; surface and borders nodular.	Scattering tubercles in liver; large colon adherent to dorsal wall.	Spleen: 35 Regional lymph node: 15 Kidney: 8 Lung: 7 Liver: 0 Uterus: 0	+

highest toward the 4th week. In animals killed later the number appears to diminish gradually. On the other hand, the lesions manifest to the naked eye appear to become more conspicuous and widespread as the number of bacteria tends to decline. One might therefore venture the general statement that for a diagnosis based on the isolation of *Bacillus abortus*, guinea pigs should be killed between the 3rd and the 4th week. For a diagnosis based on characteristic lesions they should be killed later, preferably after 7 or 8 weeks. These statements do not hold rigidly, since the progress of the disease depends largely on the dosage of the virus injected and cases have been found in this laboratory in which extensive lesions were found within 4 weeks. Table IV shows that in most instances the spleens become quite large in 4 weeks.

Series IV.

The material used in this series was from several different sources. The inoculated guinea pigs were handed over to me by Dr. Smith, who had already obtained cultures of *Bacillus abortus* directly from the material, and who inferred, therefore, that most if not all of the guinea pigs would yield positive cultures. The material came from six different cases and included fifteen guinea pigs (Table IV). They were autopsied in the 5th week. The fetal membranes of Cows 203 and 210 came from presumably full time calves. Fetuses 205a and 205b were twins from Cow 205 from which amniotic fluid had been collected at the time of delivery. Fetus 206 was of uncertain age, 28 inches long and probably between 7 and 8 months old.

The negative outcome of inoculations of meconium as compared with the positive results of inoculations of the contents of the fourth stomach should be noted. I am informed by Dr. Smith that direct cultures from meconium of Fetus 205a were positive, but the colonies were very scarce. The same was true of cultures from meconium of Fetus 205b. In these instances, therefore, direct cultures from the fetus were more reliable than guinea pig inoculations.

Series V.

This lot of seven male guinea pigs was injected with gradually increasing dilutions of a fresh culture of *Bacillus abortus*. The object of

TABLE IV

Results of Inoculation of Guinea Pigs with Fresh Material from Six Different Sources.

Guinea pig No.	Sex.	Source of material.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
				days	*gm.*				
31	Male.	Salt solution suspension (2 cc.) of meconium of Fetus 200.	Intraperitoneal.	31	+141	Slightly enlarged; nodular surface.	Minute necrotic foci in liver; hyperemia.	Spleen: 105 Liver: 0 Testicle: 0	+
32	"	1.5 cc. of thick salt solution suspension of 4th stomach contents of Fetus 200.	"	31	+106	Twice normal size surface quite nodular.	Fatty liver; necrosis of epididymis of both testicles; injection of vessels.	Spleen: 150 Testicle: countless. Liver: 0	+
33	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 203.	Subcutaneous.	33	+111	Slightly enlarged; congested and dark.	Regional lymph nodes swollen; small abscess at injection site.	Spleen: 46 Regional lymph node: 75 Liver: 0	+
34	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 203.	"	33	+165	Twice normal size; congested; faintly nodular.	—	Spleen: 90 Liver: 0 Testicle: 0	+

35	Male.	1 cc. of sterile amniotic fluid of Cow 205.	Subcutaneous.	29	+181	Twice normal size; congested; surface nodular.	Minute scattering tubercles in liver.	Spleen: 50 Regional lymph node: 60 Testicle: 0 Liver: 0	+
36	"	2 cc. of salt solution suspension of amniotic fluid of Cow 205.	"	31	+109	Four times normal size; severe congestion; smooth, easily torn surface.	Minute scattering tubercles in liver.	Spleen: 70 Regional lymph node: 80 Liver: contaminated.	+
37	"	1 cc. of salt solution suspension of meconium of Fetus 205a.	Intraperitoneal.	29	+129	Normal.	—	Spleen: 0 Liver: 0 Testicle: 0	−
38	"	4th stomach contents of Fetus 205a 0.5 cc. + 0.5 cc. of salt solution.	"	30	+25	Twice normal size; surface nodular; nodules numerous.	Minute scattering tubercles in liver.	Spleen: 18 Testicle: 6 Liver: 0	+
39	"	4th stomach contents of Fetus 205b 0.5 cc. + 0.5 cc. of salt solution.	"	33	+80	Four times normal size; marked congestion; nodular surface.	Disintegration of epididymis of both testicles; vessels injected.	Spleen: 16 Testicle: countless. Liver: 0	+
40	"	1 cc. of salt solution suspension of meconium of Fetus 205b.	"	33	+139	Twice normal size; nodular surface.	—	Spleen: 0 Testicle: 0 Liver: 0	−
41	"	4th stomach contents of Fetus 206 in salt solution.	Subcutaneous.	30	+70	Three times normal size; surface nodular.	Minute necrotic foci in liver; testicles congested.	Spleen: 350 Testicle: 0 Liver: 0	+

TABLE IV—*Concluded.*

Guinea pig No.	Sex.	Source of material.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight +. Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
				days	*gm.*				
42	Male.	1.5 cc. of salt solution suspension of lung of Fetus 206 ground with sand.	Subcutaneous.	30	+114	Three times normal size; markedly congested; easily torn.	Minute scattering tubercles in liver.	Spleen: 150 Liver: 2 Testicle: 0	+
43	"	0.6 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+72	Five times normal size; congested; surface nodular; nodules prominent.	Minute tubercles in liver. Epididymis of both testicles disintegrated; abscess in left testicle; congestion.	Spleen: thin film. Liver: 150 Testicle: 2	+
44	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+208	Eight times normal size; congested; distended capsule; easily torn.	Numerous minute tubercles in liver.	Spleen: 80 Liver: 1 Testicle: 0	+
45	"	0.5 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+162	Five times normal size; congested; nodular surface; nodules numerous.	Few minute tubercles in liver.	Spleen: 70 Liver: 1 Testicle: countless. Regional lymph node: 17	+

the experiment was to determine whether or not there is a marked difference in the gross lesions produced, and in the number of organisms present, indicated by colonies found in cultures prepared from the spleens, in the guinea pigs injected with low dilutions and those injected with high dilutions. In other words, I wished to find any difference in the severity of the disease in guinea pigs inoculated with material heavily loaded with *Bacillus abortus*, and in those inoculated with mildly infected material, all animals being autopsied within the 4th week after injection.

The culture was obtained from Dr. Smith, who had recovered it from Cow 214, after passage through a guinea pig. A 72 hour agar slant was washed off with 2 cc. of sterile salt solution. After removal to a sterile tube the material was diluted to a comparative density of a 24 hour bouillon culture of *Bacillus typhosus*. 1 : 50 of this original dilution was injected into the first guinea pig, and the dilution doubled for each succeeding animal to the seventh, which received a dilution of 1 : 3,200. All the guinea pigs were injected into the peritoneal cavity. Table V is a summary of the results. Owing to an error, cultures prepared from organs of Guinea Pigs 50 and 52 were both labeled Guinea Pig 50; therefore a definite statement is impossible, but there was an average of about 100 colonies from the spleens of both guinea pigs.

The difference in the number of colonies present in the organs of inoculated guinea pigs, indicated by colonies counted on agar slants, is not marked between those receiving injections of heavy and light suspensions of the bacillus of abortion. It is of considerable significance, however, that positive cultures can be obtained from the organs between the 3rd and the 4th weeks after inoculation with material that harbors very few organisms.

Series VI.

In order to compare the relation of the lesions and growth of cultures prepared from inoculated guinea pigs autopsied 4 weeks after injection and those kept for 4 months, the three animals given in Table VI were chloroformed and examined. Guinea Pig 53 showed no characteristic lesions, and the bacterial count was low. Guinea Pigs 54

TABLE V.

Results of Inoculation of Guinea Pigs with a Pure Culture of B. abortus, Isolated through Guinea Pigs from Cow 214, the Number of Organisms Injected Being Gradually Diminished by Increasing the Dilution.

Guinea pig No.	Sex.	Dose injected.	Length of time after inoculation when guinea pig was killed.	Gain in weight +; Loss in weight −.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
46	Male.	1 cc. of 1:50 the original dilution.	23	+	Possibly very slight increase in bulk; hyperemic; nodular surface.	Minute necrotic foci in liver; spermatic vessels injected.	Spleen: 170 Retrogastric lymph node: 100	+
47	"	1 cc. of 1:100 the original dilution.	23	+154	Twice normal bulk; mild congestion; surface nodular.	Few minute necrotic foci in liver.	Spleen: countless. Retrogastric lymph node: 48	+
48	"	1 cc. of 1:200 the original dilution.	23	+105	Hyperemic; no enlargement.	Few minute necrotic foci in liver.	Spleen: 50 Retrogastric lymph node: 15	+
49	"	1 cc. of 1:400 the original dilution.	23	+152	Twice normal bulk; congested.	Liver slightly enlarged; few minute necrotic foci. Spermatic vessels injected.	Spleen: 215 Retrogastric lymph node: 25	+
50	"	1 cc. of 1:800 the original dilution.	23	+167	Slight enlargement; hyperemic.	Two or three minute yellowish foci in liver; kidneys very dark.	Colonies present in all cultures.	+
51	"	1 cc. of 1:1,600 the original dilution.	23	+142	Normal.	Small intestine congested.	Spleen: 140 Retrogastric lymph node: 125	+
52	"	1 cc. of 1:3,200 the original dilution.	23	+92	Hyperemic.	A few minute yellowish areas in liver.	Colonies present in all cultures.	+

TABLE VI.

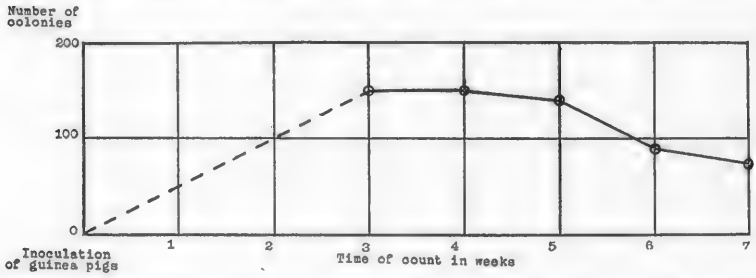
Results of Inoculation of Three Guinea Pigs with Fresh Material from Different Sources. Autopsies Performed about 4 Months after Injection.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + / Loss in weight −	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
			days	*gm.*				
53	Male.	Intraperitoneal.	123	+293	Twice normal bulk; hyperemic; surface faintly nodular.	Liver dark; few small circumscribed tubercles. Vessels supplying testicles injected.	Spleen: 16 Liver: 1 Testicle: 0 Kidney: 0	+
54	"	"	118	+215	Bulk increased at least ten times; very dark; surface nodular and easily torn.	Few minute tubercles in liver. Induration of epididymis of both testicles; small necrotic center to the mass.	Spleen: 45 Liver: 2 Testicle: 0 Kidney: 0	+
55	"	"	110	+291	Bulk increased at least ten times; severe congestion; surface nodular; capsule easily torn.	Few minute circumscribed tubercles in liver. Atrophy of testicles; induration of epididymis; vessels injected.	Spleen: 90 Liver: 2 Testicle: Kidney: 0	+

and 55 presented the characteristic enormously enlarged, nodular spleen, atrophy of the testicles, and induration of the epididymis. In all three the number of colonies, counted in agar slant cultures of the spleen, was less than those counted on cultures prepared from guinea pigs autopsied between the 3rd and 4th weeks after inoculation.

Analysis of Results.

The foregoing experiments tend to confirm earlier work in demonstrating an inoculation disease in guinea pigs due to *Bacillus abortus* of Bang which is regularly associated with an enlarged, congested spleen. Other less constant lesions affect the testicles, kidneys, and bones.



The broken line indicates no count made; the solid portion indicates the beginning of the count.

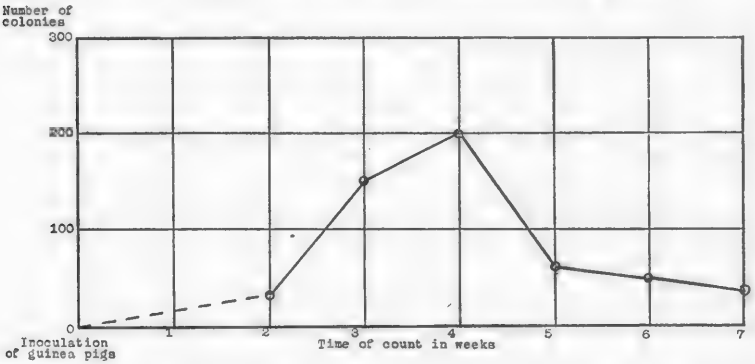
TEXT-FIG. 1. Guinea pigs of Series I inoculated with *B. abortus* 86. Colonies counted in cultures from spleens.

Minute foci frequently occur in the liver. There is, as a rule, no local lesion after subcutaneous inoculation. The animal regularly gains in weight. There is no appreciable difference in the result, whether the infection is introduced subcutaneously or into the abdomen. Nor does the size of the infecting dose within certain limits affect the result. Amounts varying between a standard dose and one-sixty-fourth of the same produced nearly the same results.

The immediate object of the investigation was to determine how far the incubation period in the guinea pig could be shortened. The results given above and Text-figs. 1 and 2 show that cultures of *Bacillus abortus* are regularly recovered from inoculated guinea pigs within

3 to 4 weeks. The figures obtained show that the number of living bacteria in the spleen of the guinea pig is larger at that time than later, although the macroscopic lesions tend to become more prominent as the bacteria decline. It remains to be seen whether the period cannot be shortened still more.

At least two or three culture tubes should be inoculated with bits of spleen tissue. In the series described such cultures were successful in 53 out of 55 cases. The two negative inoculations came from guinea pigs not affected with the disease; *i.e.*, they did not receive *Bacillus abortus* in the material inoculated. For diagnostic purposes there is no need of inoculating tubes from organs other than the spleen.



TEXT-FIG. 2. Guinea pigs of Series III inoculated with Placenta 146. Colonies counted in cultures from spleens.

CONCLUSIONS.

1. *Bacillus abortus* Bang can be regularly recovered from guinea pigs inoculated with material containing the bacillus within 3 to 4 weeks.

2. The method is especially useful in recovering the organism from fetal membranes which, as a rule, are obtained after having come in contact with fecal matter, bedding, etc.

3. The spleen is the organ in which the bacteria are regularly present and in largest numbers. Cultures must be made from it to ensure success.

NEW THORACIC MURMURS, WITH TWO NEW INSTRUMENTS, THE REFRACTOSCOPE AND THE PARTIAL STETHOSCOPE.

By FREDERICK D. PARKER, M.D.

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PLATES 58 TO 60.

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Laënnec, during his office hours one afternoon, was obliged to examine a ponderously fat patient.

"I rolled a quire of paper into a kind of cylinder, and applied one end of it to the region of the heart and the other to the ear, and was not a little surprised and pleased to find that I could thereby perceive the action of the heart in a manner much more clear and distinct than I had ever been able to do by immediate application of the ear."[1]

Thus, the first stethoscope came into existence, a hundred years ago.

Laënnec's earlier conception of sound conduction led him later to experiment with solid and hollow pieces of wood as media. The paper roll was translated into boxwood. At first these stethoscopes were thick and clumsy, without either an aural or pectoral piece, and their introduction into general use was by no means rapid. Piorry at a later date reduced the size of the stem to the thickness of a finger. Then a pectoral end appeared, and soon an aural piece followed. The flexible monaural came next, and not until 1851 was the binaural stethoscope invented. This was a product of Cammann.

There followed an era of discussion, in which we meet the names of Skoda, Beau, Zamminer, Seitz, Williams, and others. The public was swayed first in one direction, then in another. Through these years, however, a slow but definite progress in interpretation was noticeable. It remained for Flint, about 1883, to give the profession the most comprehensible explanation of breath sounds it had yet received. But the stethoscope itself has not essentially been improved since Laënnec's time. Nor can the interpretation of Flint be regarded as at all complete.

Laws Governing Sound.

Sound is a mode of motion. It consists of periodic vibrations which are complete, and which require for their transmission some suitable elastic medium. The particles of the elastic medium receive the first

[1] Quoted from Forbes (1830), p. 5.

impulse from the area of condensation adjacent. This area in turn becomes a place of rarefaction, and accordingly, the wave length is the distance from one rarefaction or condensation to the next portion of the medium in the same phase.

Of the many influences which modify the intensity of sound, there are four which, perhaps, should be mentioned. (1) The intensity of sound is inversely as the square of the distance of the sounding body from the ear. (2) The intensity of sounds of the same pitch is proportional to the square of the amplitude of the sonorous body. (3) The intensity of sound depends on the density of the air at the place at which it is produced; and this density depends upon the proportion of carbon dioxide present. (4) Lastly, sound may be strengthened by the neighborhood of a suitable sonorous body.

The laws of reflection and refraction of sound are the same as those for light and radiant heat and may be demonstrated by similar experiments. Sondhauss constructed lenses of carbon dioxide, the margins of which were limited by thin india-rubber, and he was thus able to produce either convergence or divergence of the sound waves, or bring them to a focus at will.

Diffraction of sound is the phenomenon observed when an object intervenes between a sound and the auditor. The sound waves are bent around the object, and under some conditions a sound shadow is produced. A sound shadow is the diminished perception of sound resulting from the diffraction of sound waves around a body opaque to sound vibrations. It is analogous to a penumbra.

Differences in sound are distinguished by the three properties—pitch, loudness, and quality.

"Since we are immediately conscious of the acuteness of the bird-notes, as compared with the gravity of the tone of the waterfall, we distinguish between the two sounds primarily by their difference in pitch. But before we can separate the tone of the waterfall from that of the saw-mill, we must note their difference in quality, since their pitches are nearly alike. Finally, we conclude that the bird is close at hand, but that the other two objects are a mile or more away, from the proportional loudness with which the individual sounds reach us."[2]

When any given note is sounded on most musical instruments, not that note alone is produced, but a series of other notes, of small and

[2] Hamilton, p. 24.

varying intensity. If C, which may be called a primary note, has a frequency which is denoted by unity, the whole series is given by the numbers, 1, 2, 3, 4, 5, 6, 7, etc.; in other words, first the primary C is sounded, then its octave becomes audible, then the fifth to that octave, then the second octave, then the third, fifth, and a note between the sixth and seventh to the second octave, and so on. These secondary notes are called the harmonics of the primary note. The tones which combine to produce the total effect from a single sounding body, are called partials; the lowest of these is the fundamental, while the others are called the upper partials.

If a tuning fork, having a vibration rate of 522 beats per second, is sounded, and another tuning fork having the same vibration rate is brought near it, the second fork will be caused to vibrate in sympathy with the first. The tone of one will be strengthened, and the sound waves will be of the same natural frequency. This capacity to excite vibrations in a resting body is called consonance.[3] Again, if two forks, each having a different vibration rate, are set in oscillation and are caused to approach each other, their phases will interfere, and undulations in intensity, called beats, will occur. The number of beats per second equals the difference between the vibration numbers of the sounds. Dissonance is produced by this phenomenon. We shall see later with what importance these phenomena affect our auscultatory signs.

Von Helmholtz and König have simplified our understanding of sound analysis and synthesis by constructing small globes or resonators with aural attachments. A whole series of resonators may be used in listening to sound reinforcement of partials or their combinations in pure or compound tones. Sound reinforcement may be demonstrated by lowering the pectoral end of a stethoscope into a hollow vase and noting the reinforcement of one or perhaps two tones as struck upon the pianoforte. The roar of the sea-shell beside the ear is due to its capacity to reinforce the infinite number of small tones and partials that are ordinarily moving through the atmosphere, but which are imperceptible to the ear.

[3] We must not confuse overtones derived from thoracic murmurs with those which are always added by the instrument employed, and which are peculiar to its capacity in tone production.

It is to these laws that we must turn for an explanation of respiratory murmurs and heart sounds.

Sound Interference and the Laws Governing Closed Pipes.

If a tuning fork is struck and twirled beside the ear, it will be noticed that in its passage of revolution four places will occur where silent areas appear to exist. These are nodal lines of silence. They are due to the propagation outward of two sets of vibrations which have the same frequency, but which are in opposite phases. Upon vibrating plates and membranes the nodal lines which are formed vary with an alteration in pitch. They may readily be observed by experiment, when light powder is sifted upon a vibrating plate.

As distinct from open pipes, pipes which are closed at the end opposite the mouthpiece, and which have a fundamental tone represented by 1, may be caused to give forth successively the notes, 3, 5, 7, 9, etc.; that is, only the odd harmonics of the primary note. Notwithstanding that the above laws are enunciated with reference to an organ pipe, they are true of any other closed pipe of uniform section. When an air column contained within a pipe is set into vibration, nodes and loops occur. By a node must be understood a section of the column of air contained in the pipe, where the particles remain at rest, but where there are rapid alterations of condensation and rarefaction. By a loop must be understood a section of the column of air contained in the pipe where the vibrations of the particles of air have the greatest amplitudes, and where there is no change of density. In the case of a closed pipe, the top is always a node. And upon the division of the pipe into nodes and loops depends the formation of the number of partials.

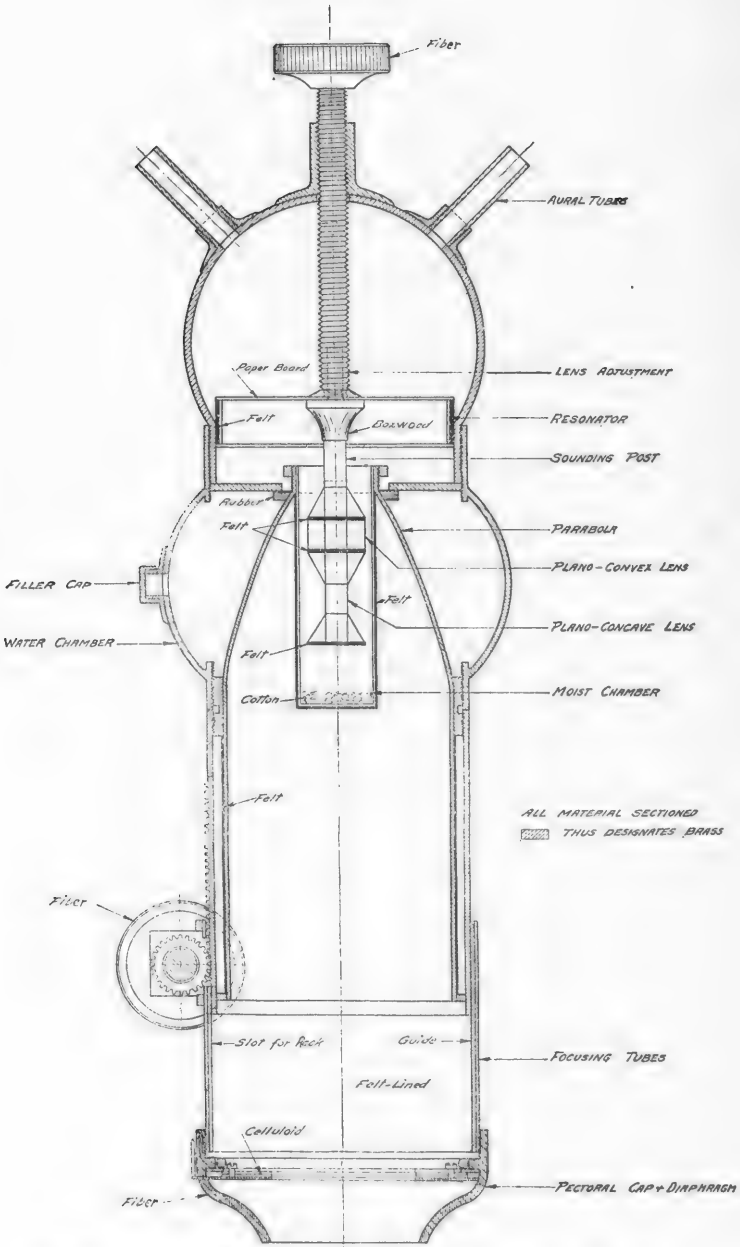
My early impressions of the complexity of acoustics and their relation to thoracic murmurs soon led me to realize the futility of their further study with the ordinary stethoscope, for within the compass of the thorax it will be seen that there exist the most complicated compound tones. Some of these are blurred to our perception by variations in their intensity or by sounds of a relatively greater intensity which overshadow them. Of the pure tones, some depend for their existence upon pipes, others upon resonating cavities, while

the vibration of solid structures lends quality to a third class. We have also to consider that these tones in the chest are modified finally by their transmission to a series of vibrating membranes which definitely have a capacity for their reflection and refraction in their propagation outward to the integument. It must be understood in this regard that our faculty of perceiving sounds depends rather on their intensity than on their pitch, and that of the audible sounds, we have a variation between 16 vibrations a second and from 16,000 to 20,000 for the higher frequencies, the wave lengths of which follow in a definite ratio.

Refractoscope.

With these facts before me, I began to conceive the design of an instrument especially adapted for this acoustic study. During the progress of the experimental work I found it necessary to build several. The mechanics of the refractoscope (Fig. 1), as I have called it, in its relation to sound waves are described briefly below. The use of this instrument led to the discovery of the existence of sound shadows within the human thorax.

The vertical section of the refractoscope (Text-fig. 1) will give an idea of its shape and size. It consists of a series of tubes, straight below, and globe-shaped above. The parabola portion of this instrument is the conic section embodied in the upper part of the innermost tube (Fig. 2). It is symmetrical and has a fixed focal distance as a reflector, and within this parabola at the focal end are three compound wooden lenses. Its inner surface is polished nickel, and its outer portion is encased in felt. Surrounding the parabola proper is a water chamber, since by experiment it was observed that without the water chamber the instrument is less sensitive to some vibrations under observation. Surrounding the inner tube is an outer movable tube, actuated by a rack and pinion. By this adjustment the listener is able to focus down upon a sound in a manner analogous to focusing a microscope. The sound is thereby reinforced through resonance. The pectoral cap is provided with an iris diaphragm, the movement of which is a finer adjustment for changing the ratio of the length to that of the bore of the inner tube. Both of these adjustments have graduated scales.



TEXT-FIG. 1. A vertical section of the refractoscope. Scale one-third.

Above the water chamber is a second chamber containing six boxwood lenses, a sounding post, and a resonator. The lenses rotate about a vertical axis, and are actuated either up or down by a screw projecting from the top of the instrument. The six lenses oppose each other on the perpendicular sides of the sounding post. The cross-section of this post is an equilateral triangle, and is so devised to lessen interference. Each lens is subdivided into three prisms with felt mutes interposed between adjacent surfaces; the prisms when placed together form the six lenses, and three of these are plano-convex and three are plano-concave. A moist chamber is placed in the top of the parabola, and vertical slits in the sides of the chamber, made at the top, permit the passage of sound waves as they are brought to a focus upon the lenses. The outer surfaces of the moist chamber are covered with felt, in order that the tube harmonics may be muted, as far as possible. It may now be readily seen if harmonics represented by the series 3, 5, 7, 9 are reinforced and caused to impinge upon the hypotenuse of one prism, that during their refraction, the separate upper partials will penetrate to the sounding post, or be muted in the felt, depending upon their intensity, and upon their direction towards the apex or base of the prism. Ordinary rubber stethoscope tubes complete the instrument in its present development.

That this principle of so refracting sound is fallacious in theory—except for instances of the higher frequencies—was demonstrated by the incapacity of the lenses as such, to modify the larger wave lengths which I desired to study. In order, therefore, to perceive intelligently waves of from $\frac{1}{2}$ to $1\frac{1}{2}$ inches in length, the lenses required would necessarily need to be from 4 to 6 inches in diameter, or even larger. This size, necessarily large for deeper tones, impairs the instrument for ordinary practice. But the refractoscope was not without important teachings. The first precept of interest and importance to instrument construction was that no instrument is capable of being placed upon the chest wall without conveying to the sense of hearing sounds dependent upon its capacity as a conductor, by virtue of its solid parts. The second precept was quite unexpected. It was found that the conductivity of the instrument was altered through the law of resonance, by a slight adjustment of the lenses. Upon this discovery were based the subsequent designs for the partial stethoscope, described below.

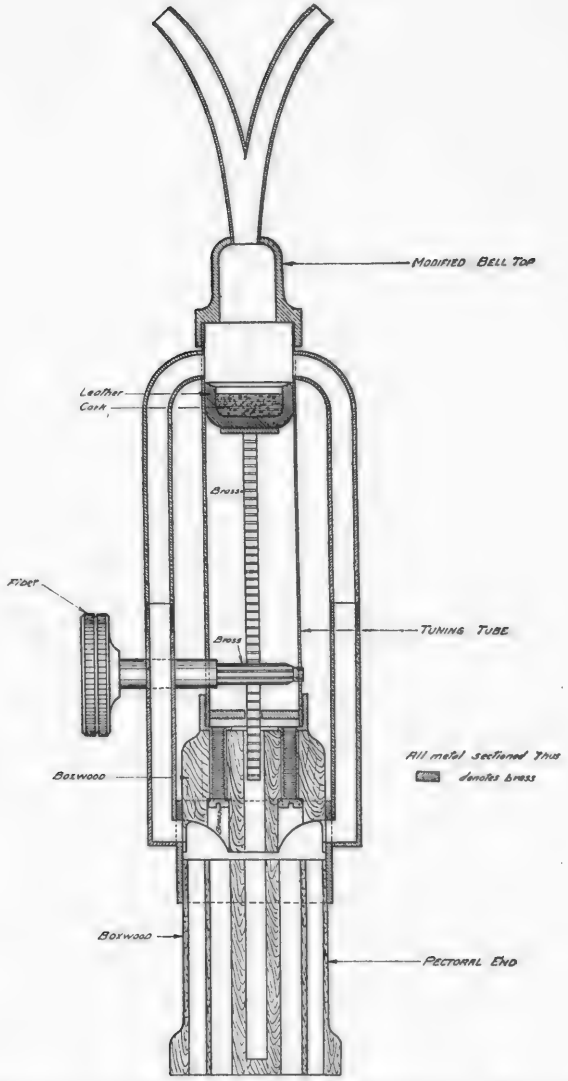
Partial Stethoscope.

The partial stethoscope (Fig. 3 and Text-fig. 2), depends for the most part upon its property to communicate sound vibrations by virtue of its solid parts. This is an improvement over air conduction instruments, for if it is true that the faculty for perceiving the higher frequency vibrations is dependent upon their intensity of transmission, it will follow that certain sounds will not be heard, if we depend upon air conduction as a sole means of transmission.

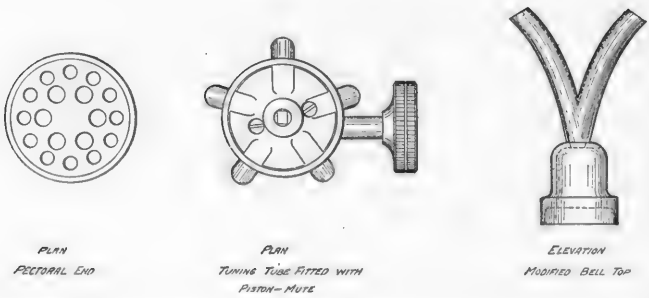
Sound perception may be altered with the partial stethoscope by (1) any variation in the conductivity of the instrument; (2) any alteration in the amplitude of sound vibrations occurring within the air chambers of the instrument.

The conductivity of this instrument may be altered by an adjustment of the thumb nut. The amplitude of vibration may be increased by placing a layer of blotting-paper between the pectoral end and the sounding body,—this increases both loudness and intensity,—and also by lessening the pressure contact of the instrument against the sounding body. The amplitude of vibration may be decreased by increasing the pressure contact, or by removing the instrument altogether.

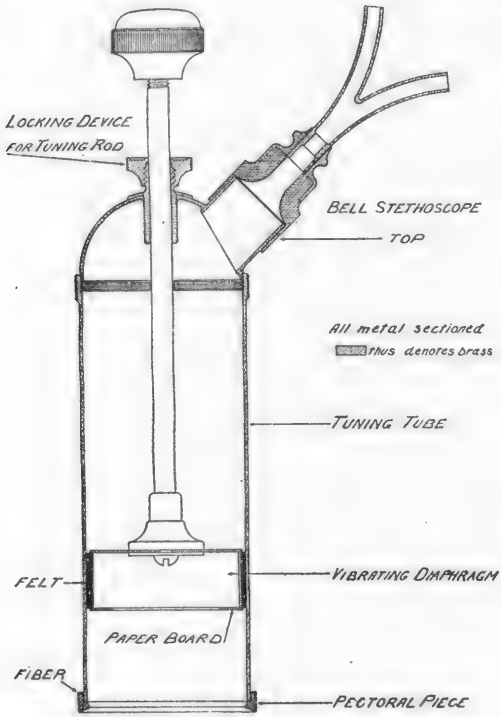
Partials may be studied by reinforcing the beginning or the ending of the chain, at the expense of that portion which is less desired. It should be understood that this maxim refers to tones which have a measurable duration, readily perceived and comparable with tones of a shorter duration. It is interesting to note that when two tones which are constant in pitch and in rhythm seem to change that rhythm in their cycle or to be timed differently, it appears to the sense of hearing that the partials of the one tone have either been anticipated, or that the partials of the other have been followed, or *vice versa*. It results accordingly that adjustments which increase the pitch of a fundamental mean that the partials of it are heard to follow its original time, while adjustments which decrease the pitch of a tone mean that the partials of it are heard to anticipate its original time. The lowest partial is the fundamental, while those above it in pitch are partials in that series.



TEXT-FIG. 2. A vertical section of the partial stethoscope. Scale one-half.



TEXT-FIG. 3. The partial stethoscope, showing the multiple air chambers within the pectoral piece, and the usual Bell stethoscope, modified and forming the top of this instrument. Scale one-half.



TEXT-FIG. 4. A variation of the partial stethoscope, with a vibrating piston-mute. Scale one-half.

Text-fig. 3 shows another view of the partial stethoscope. The lower half is made of a perforated piece of boxwood, while the upper half is a hollow tube containing a piston-mute. Both air chambers contained within these parts are connected by five hollow tubes, and in the conduction of sound to the top of the instrument, by virtue of these shell tubes, the air spaces within are set into vibration, transforming the cavities into a sonorous body of air, which is perceived as sound. By regulating the position of the piston-mute, sound partials desired for study may be reinforced. By a careful variation of the amplitude of sound under observation the listener is still further able to pick out, focus upon, or mute sounds at will.

The vertical section (Text-fig. 4) shows a partial stethoscope having a vibrating diaphragm, which is nothing more nor less than a piston-mute. The amplitude of sounds with this instrument is somewhat increased over the less sensitive model.

Results Obtained with the Instruments.

With these instruments the following facts have been determined.

The sounds heard through the refractoscope and through the partial stethoscope are different in many respects from those we hear in ordinary auscultation.

There exist sound shadows, of which we have been ignorant, and of these there are two varieties, respiratory sound shadows and cardiac sound shadows.

Heart sounds appear to take on the characteristics of chamber sounds in a degree greater than we have realized; and dilated hearts with poor muscle respond to more harmonics than does muscle with better tonus.

The so called third heart sound, noted by many observers and discussed by both Barié and Thayer, does exist as a true heart sound. It is the sound produced during auricular systole and is heard best at the apex.

There is a cardiac sound which I shall call the outflow sound. The heart is a tube with resonant walls. These walls are set in vibration: (1) while the heart is filling; (2) when the auricle contracts; (3) when the ventricle contracts, thus causing the valves to vibrate;

(4) while the ventricle is being emptied; (5) when the semilunar valves close. The flow of blood into the heart is too gentle to cause vibrations sufficient to give rise to audible sound. The second or auricular vibration causes the sound of Barié and Thayer. The third sound is that now termed clinically the first sound. The fourth is my outflow sound. The fifth is the so called second sound. The outflow sound is heard between the first and second valve sounds of the heart. During this period the outflow sound does not appreciably vary in intensity, but it noticeably rises in pitch. When the ventricle shoots its considerable mass of blood into the great arteries, it is *a priori* probable that the rushing flood would set the elastic walls in vibration. The outflow sound cannot be a continuation of the so called first sound, because the intensity of the outflow sound does not perceptibly diminish.

Vesicular breathing and bronchial breathing have the same origin, but in vesicular breathing there are vibrations added by the parenchyma of the lungs, and in bronchial breathing there are fewer partials muted.

The ratio of inspiration to expiration during any one phase is dependent upon the domination of one tone with its chain of partials over the other,—by reason of improved or impaired resonance in that phase, and this may be altered somewhat, by reinforcing the other tone or chain.

There are lines of diminishable intensity emanating from the integument during the phases of respiration. They are transient in character and may be influenced by pressure exerted upon the chest wall from without.

In all sounds emanating from the thorax the pleuræ are in a state of vibration.

Some sounds produced in the more remote parts of the chest are brought to a focus in the apices of the lungs.

CONCLUSIONS.

1. An understanding of the physics of sound is essential for a better comprehension of refined auscultation, tone analysis, and the use of these instruments.

2. The detection of variations of the third heart sound should prove a valuable aid in predicting mitral disease.

3. The variations of the outflow sound should prove a valuable aid in determining early aortic lesions with the type of accompanying intimal changes.

4. The character of chamber timbre as distinct from loudness heard as the first and second heart sounds denotes more often the condition of heart muscle, and must not be confounded with valvular disease.

5. The full significance of sound shadows is uncertain. Cardiac sound shadows appear normally in the right axilla and below the left clavicle. Their mode of production is quite clear.

6. Both the third heart sound and the outflow sound may be heard with the ordinary stethoscope.

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EXPLANATION OF PLATES.

PLATE 58.

FIG. 1. The refractoscope.

PLATE 59.

FIG. 2. The refractoscope assembly. 1. The tubes used in tuning. 2. The lenses, sounding post, and resonator. 3. The reflecting parabola encased in felt. 4. Water chamber. 5. The pectoral cap, with iris diaphragm.

PLATE 60.

FIG. 3. The partial stethoscope.

FIG. 1.

(Parker: New thoracic murmurs.)

FIG. 2.

(Parker: New thoracic murmurs.)

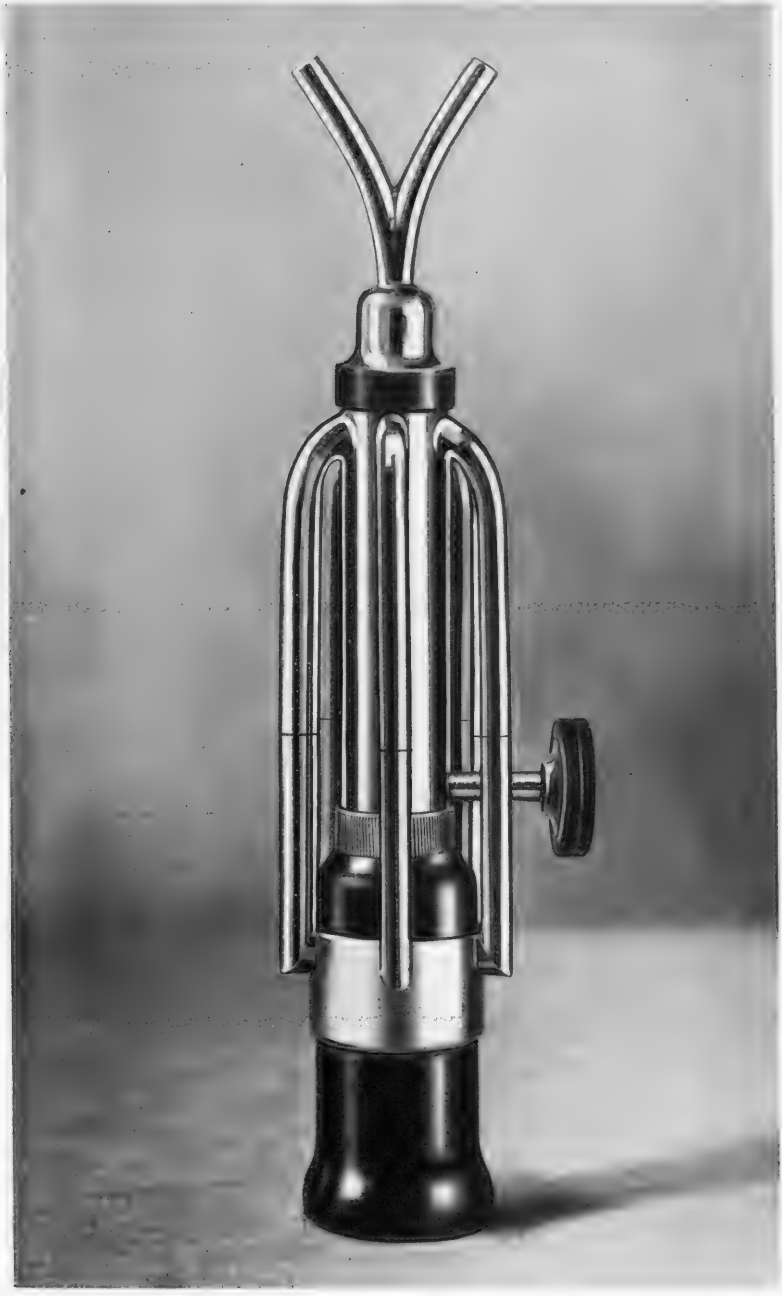

FIG. 3.

(Parker: New thoracic murmurs.)

A NEW TYPE OF SYRINGE, ESPECIALLY ADAPTED FOR INTRAVENOUS INJECTIONS OR THE ASPIRATION OF BLOOD.

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Plates 61 to 63.

(Received for publication, September 4, 1918.)

During the present war The Rockefeller Institute resumed the production of antimeningococcus serum and increased its output of antipneumococcus serum. At the entrance of the United States into the struggle the Institute undertook the production of these sera in much larger quantity than previously. The imunization of a large number of horses introduced many technical problems which were of relatively little importance in the immunization of only a few animals. Among these problems was that of a satisfactory syringe.

In the immunization of horses against meningococci and pneumococci fresh living cultures are injected intravenously. The amount of material injected is usually from 15 to 30 cc. For this purpose syringes made entirely of glass have been used. Their desirable features are (1) simplicity and the ease with which they may be cleaned, (2) the convenient slip joint for attaching the needle, and (3) the smoothly running ground glass plunger enabling one to sense by the pressure whether the material is being injected into or outside the vein. The objections to syringes of this type are (1) their perishability as a result of accident or of frequent boiling, (2) the difficulty of dislodging foam or bubbles after drawing up material into the syringe and the consequent danger of injecting air, and (3) the liability to lose material or to draw in air because of the ease with which the plunger moves. The last objection does not apply to the small syringes made entirely of glass but the weight of the contents or of the plunger in the larger syringes (25 cc.) is sufficient to move the plunger against the friction

of the binding spring supplied with these syringes. The conditions are not ideal when one is approaching a nervous horse.

Because of these difficulties there was much loss of time, breakage of syringes, and inaccuracy of dosage—difficulties which were serious for the work in hand and of constant occurrence. Catalogues of manufacturers and supply houses were searched and many kinds of syringes were tried; others could not be obtained readily because of war conditions. Many were too intricate, difficult to clean, to keep in repair, or easily broken, and expensive to replace. Those with expanding rubber or leather plungers worked hard, jumped, or leaked. None was so satisfactory as the syringe made entirely of glass in use.

The syringe to be described was designed and many of them have now been in use for over 6 months with good results. The barrel consists of an ordinary 50 cc. centrifuge tube (Fig. 1, *A*) or large test-tube (inside diameter $\frac{15}{16}$ to 1 inch). This is the only breakable part of the syringe. The plunger is made of metal (nickeled brass) with expanding rubber packing (Fig. 1, *D*). The outlet is through the plunger instead of at the end of the barrel. The plunger is made up as follows:

There is a core of metal tubing $6\frac{1}{2}$ inches in length (Fig. 1, *D*) with a bore $\frac{1}{16}$ inch in diameter. The upper end of the core is cut to fit a Luer needle (Fig. 1, *B*). The lower end terminates in a piece of hemispherical metal $\frac{7}{8}$ inch in diameter (Fig. 1, *D*) of suitable size to fit the bottom of the glass tube (Fig. 1, *A*). The bore of the core does not continue straight through the hemispherical lower end but communicates with a hole of equal size leading laterally from near the edge of the hemisphere (Fig. 1, *Dd*). Over the core and next to the hemispherical lower end is fitted a thick rubber washer (cut from pressure tubing, outside diameter $\frac{7}{16}$ inch), then a thick metal washer (outside diameter $\frac{5}{8}$ inch), and finally another rubber washer. The packing consists of a piece of pure gum rubber tubing (outside diameter $\frac{7}{8}$ inch, inside diameter $\frac{5}{8}$ inch) (Fig. 1, *C*) with smoothly cut edges and of a length slightly less than the total thickness of the series of washers on the core (about $\frac{5}{8}$ inch). It is essential to cut the packing from a piece of tubing with a perfectly smooth outer surface. Linen-finished tubing or tubing with a little longitudinal furrow in the line of the seam is not suitable. The packing is placed over the core around the series of washers and is centered by the metal washer. The lower edge of the packing rests against the hemisphere and the upper edge against the sleeve (Fig. 1, *F*) which is slipped on over the core after the packing is in place. Screwed onto the core above the sleeve is a wing nut (Fig. 1, *E*). As this nut is screwed down against the sleeve it causes the latter to expand the packing. There is formed between the rubber washers and the packing an air chamber the function of which will soon be described.

In use the plungers assembled as described are sterilized by boiling. The dose of material for injection is measured out by pipette into the cotton-stoppered sterile glass tube (Fig. 1, *A*). The cotton plug is discarded and the plunger is inserted into the tube (Fig. 2). The upper end of the tube is sealed by a rubber stopper with a metal bore which fits loosely around the sleeve of the plunger (Fig. 1, *Fa*). While the plunger is held by one of the milled areas around the sleeve the wing nut is screwed down until the expanding packing makes contact with the sides of the glass tube. Held in the position shown in Fig. 2 with the outlet in the hemispherical end of the plunger uppermost, bubbles of air or froth are easily expelled; hence, the reason for placing this outlet at the side rather than at the center of the hemisphere. If it is desired to leave a few small bubbles in the syringe rather than lose the small amount of material which may accompany their expulsion, they may be left in the syringe with perfect safety if during injection the syringe is turned so that the outlet in the side of the hemisphere is at the lower side of the tube; in this position the bubbles remain at the upper side and do not find the outlet. Injection may be made at any angle. In the intravenous injection of horses at this Institute it is customary to inject upwards. The needle (detached) is first inserted upwards into the jugular vein, the thumb being pressed over the jugular below the needle to cause the vein to bulge. When a small stream of blood coming through the needle shows that it is in the vein, the syringe is connected with the needle and the material injected by pushing the tube or barrel of the syringe against the plunger. The motion required is the same as that employed in pushing the plunger into the barrel of the ordinary syringe. The wing nut serves also as a finger rest (Fig. 2).

The function of the air chamber within the packing remains to be described. As mentioned above the ease with which the ground glass plungers of the syringes made entirely of glass move in the barrel is of great advantage in enabling the operator to detect by sense of touch any increase of resistance to the outflow of the material, often due to the slipping of the needle out of the vein during the injection of a restless horse. On the other hand, the rubber plungers of syringes on the market were found to work hard or jump if expanded sufficiently to prevent their leaking. With the syringe

being described the same difficulty was encountered until it was discovered that it could be remedied by leaving an air chamber within the packing and communicating freely with the interior of the syringe by a single small hole (Fig. 1, *Dc*) which runs diagonally upward through the edge of the hemisphere from a point near the outlet. The capacity of the air chamber in the syringes being used is about $\frac{1}{15}$ cubic inch. At first thought it might seem that injection material would pass into the air chamber and be wasted, but such is not the case. When during injection pressure is exerted on the bottom of the syringe tube a very small amount of material may pass into the air chamber but in so doing it compresses the air within the chamber and as soon as this pressure is relieved, as it invariably is towards the end of the injection, the drop of material is again forced out of the chamber by the expanding air. The compression of the air within the chamber serves another purpose; it causes the packing to expand automatically in response to pressure of all degrees. All that is necessary is to turn the wing nut sufficiently to cause the packing to make contact with the interior of the glass tube on all sides. In this condition the plunger moves easily and smoothly. As soon as the resistance to outflow increases, however, the packing expands and always just sufficiently to prevent leakage by the packing. In fact one may place the tip of the syringe against a rubber stopper on a table or against the wall and bear his whole weight against the bottom of the glass tube and the contents will not leak by the plunger, but if the resistance to outflow is removed the contents are expelled with the greatest ease. The ease with which the plunger moves is comparable only with that of a ground glass or metal plunger.

The syringe as above described is best adapted for injection, though it may also be used for aspiration against slight resistance. For aspiration against heavy resistance the arrangement of washers should be as shown in Fig. 3. With this arrangement the air chamber communicates with the atmospheric air through a small opening (Fig. 3, *c*) in the sleeve (closed by the upper rubber washer in Fig. 1).

If the syringe is desired to serve for both injection and aspiration against heavy resistance, it may be assembled as shown in Fig. 4, with two air chambers, the upper one expanding during aspiration and the lower one during injection.

The advantages claimed for the syringe are especially those desired for the intravenous injection of horses in large numbers where economy of time and expense, and the elimination of error are of great importance. These advantages are: (1) durability—non-breakage of expensive parts, the use of glass tubes for barrels, the use of rubber tubing for packing; (2) an easily moving, non-leakable, automatically expanding plunger; (3) the easy expulsion of air bubbles and froth; (4) the ability to measure out the doses and fill the syringes in the laboratory rather than in the stables.

As used in this Institute the syringes are filled in the laboratory, placed in the sterilizers in which the plungers have been boiled, and sent to the stables. At the stables each horse which is to be injected is turned about in the stall, tied with head outwards towards the aisle. The veterinarian with syringes, needles, absorbent cotton, and alcohol on a ward carriage moves down the aisle injecting the horses as he comes to them. The time required averages less than 2 minutes for each horse. A separate syringe is used for each horse, but it is suggested that it is possible to inject a series of animals, receiving the same material, with the same syringe plunger. The doses may be measured out into a series of the glass tubes, and the plunger moved from one tube to another for each injection. The cost of the syringe is moderate, the metal parts of those specially made for us costing less than $4.00 each.[1] The rubber packing of syringes in daily use needs to be renewed once in about 3 months. During the past 6 months over 3,000 injections have been made without serious accident due to defect of syringes.

It is suggested that there may be other uses for this type of syringe. Blood cultures might be made by aspirating blood into the tube of the syringe containing bouillon, the plunger removed, a sterile cotton plug inserted, and the tube incubated. For the collection of blood corpuscles blood might be aspirated into the tube of the syringe containing citrate solution, the plunger removed, a sterile stopper inserted,

[1] The syringes used have been ordered through George Tiemann and Company, 107 East 28th Street, New York City, and were made by Becton, Dickinson and Company, Rutherford, N. J. Suitable tubing for rubber packing has been obtained from the Scientific Materials Company, Pittsburg, Pa.

and the tube placed into the centrifuge. A slight variation of the technique would enable one to collect small samples of serum or defibrinated blood aseptically.

EXPLANATION OF PLATES.

PLATE 61.

FIG. 1. (*A*) Centrifuge tube used for barrel of syringe.

(*B*) Luer needle.

(*C*) Rubber packing cut from tubing.

(*D*) Core of plunger. (*a*) Rubber washers. (*b*) Metal washer. (*c*) Opening into air chamber. (*d*) Outlet communicating with bore of plunger.

(*E*) Wing nut and finger rest.

(*F*) Sleeve to be placed over core above packing. (*a*) Metal-lined rubber stopper for closing open end of barrel.

PLATE 62.

FIG. 2. Assembled syringe held in position for expelling air, or for injection after the air has been expelled.

PLATE 63.

FIG. 3. Arrangement of washers and packing for aspiration. (*a*) Rubber washers. (*b*) Metal washers. (*c*) Opening into air chamber.

FIG. 4. Arrangement of washers and packing for both injection and aspiration. (*a*) Rubber washers. (*b*) Metal washers. (*c*) Openings into air chambers.

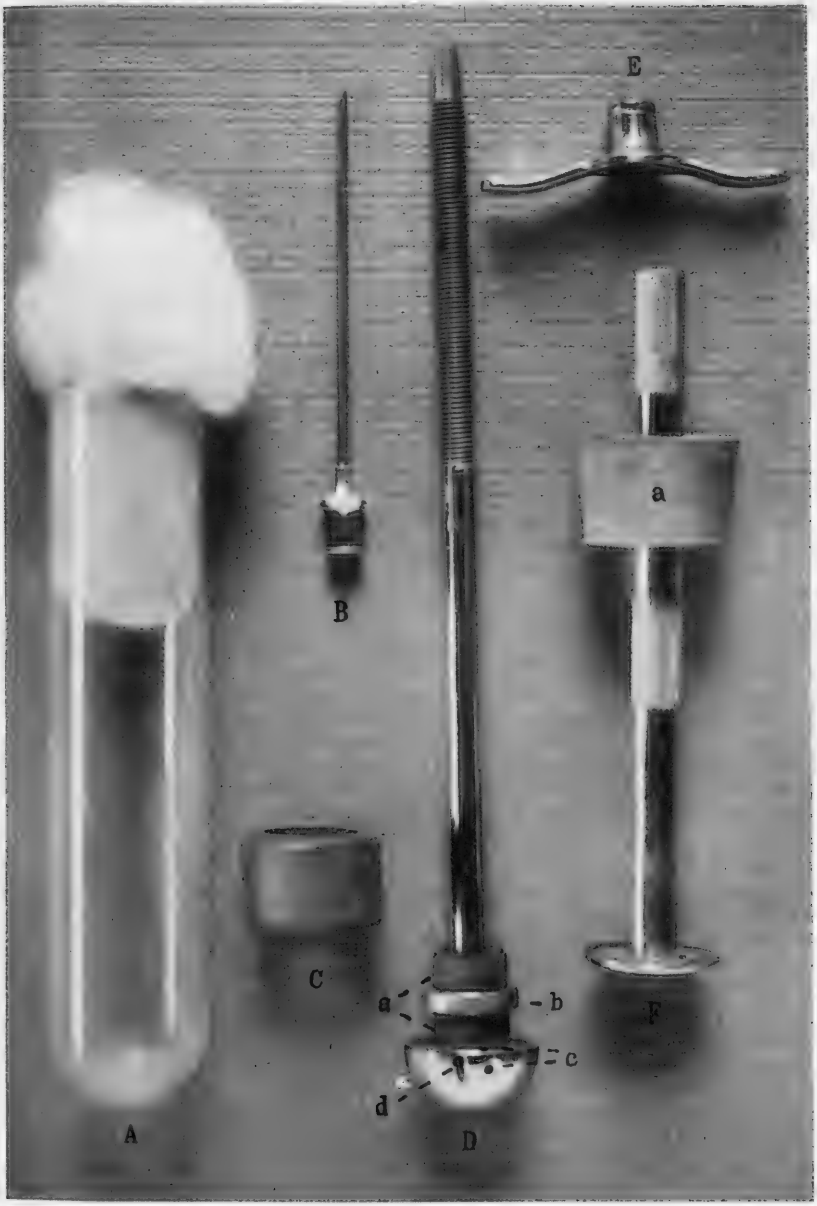

FIG. 1.

(Brown: New type of syringe.)

FIG. 2.

(Brown: New type of syringe.)

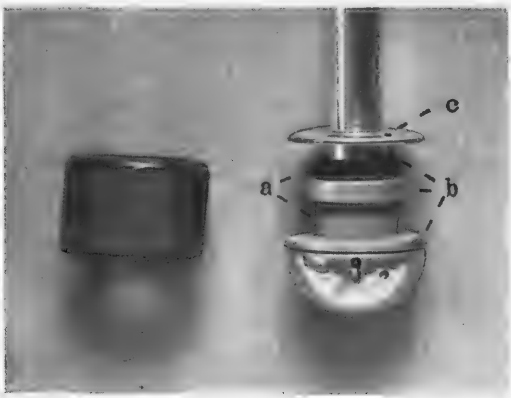

FIG. 3.

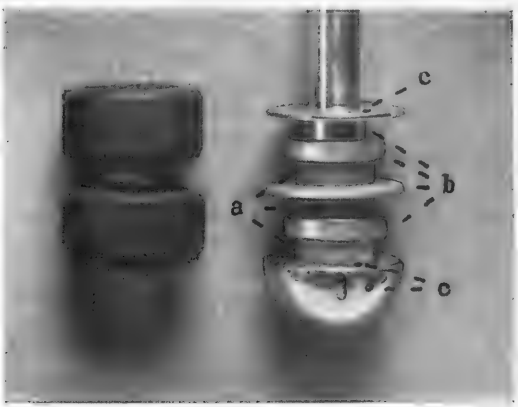

FIG. 4.

(Brown: New type of syringe.)

EXPERIMENTAL HEMOCHROMATOSIS.

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PLATES 64 TO 70.

(Received for publication, June 17, 1918.)

In pernicious anemia and hemochromatosis an iron-containing pigment, hemosiderin, ordinarily derived from hemoglobin, is deposited in organs free from such pigment in most conditions that involve blood destruction. When sudden hemolysis takes place in a previously healthy human being, as after cutaneous burns or the action of a "blood poison," hemosiderin granules appear in the spleen, the red marrow, and sometimes in certain lymph nodes, but as a rule in these organs only. A similar localization of the pigment is found in most diseases accompanied by a destruction of red cells,—acute malaria, for example; and it is regularly encountered in animals submitted to experiments or infections which, like trypanosomiasis, involve a breaking down of blood. Far different is the siderosis of pernicious anemia and hemochromatosis. The spleen and bone marrow are still pigmented, but to only a slight degree as compared with some of the other organs. In pernicious anemia most of the hemosiderin is found in the liver and much in the kidneys. In hemochromatosis the liver, kidneys, pancreas, heart, abdominal lymph nodes, and many other organs are heavily loaded with the pigment.

What is the cause for the difference thus outlined? Has the hemosiderin in hemochromatosis another source than the blood? These and other important questions suggest themselves. And numerous attempts to answer them through an experimental production of the significant lesions have already been made. Stadelmann,[1] Hunter,[2] Biondi,[3] and others obtained a siderosis of the liver parenchyma as the

[1] Stadelmann, E., *Arch. exp. Path. u. Pharmakol.*, 1887, xxiii, 427.

[2] Hunter, W., Pernicious anæmia, London, 1901, 182.

[3] Biondi, C., *Beitr. path. Anat. u. allg. Path.*, 1895, xviii, 176.

result of fatal doses of toluylenediamine. The drug injures the liver greatly. Schurig,[4] who injected pure hemoglobin into rabbits day after day for several weeks found some hemosiderin in the liver and kidneys, in addition to the usual abundance in spleen and marrow. Muir and Dunn,[5] producing an acute hemolytic anemia in rabbits by means of a specific hemolysin, noted a little granular hemosiderin in the liver and a diffuse Prussian blue reaction in the kidney parenchyma of animals surviving the destruction in the course of 3 days of more than half their total blood. Other less successful results might be quoted. The general findings warrant the conclusion that under most circumstances of blood destruction the spleen and red marrow act as depots for hemosiderin and more than suffice for the purpose; but when the breaking down of red cells is fulminant, or large quantities of blood pigment are furnished the organism for a considerable period, the buffer activity of these organs is overcome, and some little hemosiderin is laid down elsewhere. There are several obstacles to obtaining a great deposition of it by experimental means. Free hemoglobin injected in considerable amount is for the most part rapidly lost through the kidneys; blood cells that are damaged tend to accumulate in the capillaries and cause lesions; while the blood poisons, repeatedly administered, injure other tissues besides the blood, with complex results. In view of these circumstances the unsatisfactory outcome of experiments is easily understood.

Method.

It has seemed to us that better results than those described might be obtained through a utilization of the animal's own ability to break down blood under circumstances such that more pigment is liberated than spleen and marrow can deal with. A means for the work has come to hand in the method of repeated transfusion employed by one of us with Robertson for the study of normal blood destruction.[6] Rabbits rendered plethoric by the day to day injection of 10 cc. of the citrated whole blood of other rabbits soon acquire the ability to dispose

[4] Schurig, *Arch. exp. Path. u. Pharmakol.*, 1898, xli, 29.
[5] Muir, R., and Dunn, J. S., *J. Path. and Bacteriol.*, 1914–15, xix, 417.
[6] Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1916, xxv, 665.

rapidly of the foreign blood, and this in many instances without the development of demonstrable immune bodies. We have accordingly transfused eleven animals for months, injecting them 6 days out of every 7, and towards the end increasing the daily amount of blood to 15 cc. For each recipient a number of donors were used in rotation. In the early weeks some of the recipients developed weak agglutinins for the foreign blood. Later, as the injections were continued, such antibodies disappeared.

Findings in Relation to Pernicious Anemia.

In the present paper a general description of the results of the work will be given. A study of certain of its special features is to be published later.

The rabbits killed after a few weeks of plethora induced by the repeated injection of blood showed merely the lesions already described by many observers[7] as following one or two massive transfusions; namely, an enlarged spleen, turgid with cellular debris and pigment, and phagocytes full of red cells; a considerable siderosis of the marrow; and a slight one of the abdominal lymph nodes—in other words, the findings usual after blood destruction in normal animals. When the injections had been kept up for 2 months there was in addition a pigmentation of the parenchyma of the liver and kidneys with fine yellow-brown granules which on test proved iron-containing. The pigment was abundant in the peripheral cells of the hepatic lobules and in the convoluted tubules of the kidneys, as is the case in pernicious anemia. Save for the siderosis, a general plethora, and a consequent slight, central atrophy of the liver cells, the organs appeared normal. In human pernicious anemia the spleen is relatively free from pigment, whereas in our animals this organ was always most heavily loaded with it. The difference is an important one, indicating as it does either that the spleen is altered in pernicious anemia, and unable to take up the products of blood destruction, or that its activity is somehow circumvented, which is the supposition in Hunter's theory of a portal blood destruction.

[7] For instance, Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1910, xiv, 294.

Findings in Relation to Hemochromatosis.

Seven rabbits were transfused over a period of from 3 to 6½ months. The findings in these have great interest, for they are such as are associated, in human beings, with hemochromatosis and with this disease alone. Most of the animals were healthy when killed and had gained weight despite the long standing plethora which involved a day to day hemoglobin of 120 to 160 per cent Sahli, and, as the autopsies showed, a pronounced general congestion. Jaundice was not noted at any time, nor was hemoglobin or sugar found in the urine. The absence of the latter is accounted for by the state of the pancreas which showed lesions of a mild grade in contrast to the serious changes which accompany the *diabète bronzé* of man.

In comparing the rabbit material with that of human hemochromatosis, we have made use of the recent excellent descriptions of Sprunt,[8] Gaskell and his associates,[9] and also of specimens from an outspoken case of hemochromatosis studied at the Hospital of The Rockefeller Institute, and autopsied by one of us.[10] The older studies of hemochromatosis are in general marred by the use of unsatisfactory methods to demonstrate iron-containing pigment.

The changes in the rabbits were, in general, much less marked than those observed post mortem in human hemochromatosis, as would naturally follow from the fact that the pigmentary process had not gone so far in the animals as even to menace health. Nevertheless, many of their organs, notably the liver, kidney, heart, visceral lymph glands, pancreas, spleen, and marrow, had, to the naked eye, a distinct brown tint. The liver was in several instances of a light chocolate color and showed a slight, diffuse cirrhosis, indistinguishable in the gross from that so frequently intercurrent in rabbits. The spleen was never larger than after several weeks of transfusion and often smaller. The skin of a long transfused white rabbit had a brownish tone. In every case the vessels were engorged, the liver was swollen, owing to congestion, and the heart was enlarged and dilated. The histological findings were essentially similar in all the animals, differing only in degree.

[8] Sprunt, T. P., *Arch. Int. Med.*, 1911, viii, 75.

[9] Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913–14, vii, 129.

[10] Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.

Pigments.

Most of the pigment in the organs differed not at all in appearance or reactions from the hemosiderin observed in human beings. It had the form of orange-brown, moderately refractile granules, usually minute, but sometimes coarse and irregular, as in the spleen. When exposed to ammonium sulfide the granules turned black, and when tested by Nishimura's method[11] they took on a fine, deep blue color. In determining their distribution to the various organs, we have regularly used the latter method, which Sprunt and others, working with human material, have found satisfactory.

Hemofuscin, a yellow or brown pigment which fails to react for iron, occurs in quantity in some cases of human hemochromatosis, whereas in others it is practically absent. As Sprunt has pointed out, its amount varies greatly with the method used to demonstrate it. With the Nishimura test there is always far less than with Perl's reaction which the older authors generally employed for the demonstration of iron. The hemofuscin is to some extent characterized by its situation, being found especially in the capsules and trabeculæ of lymph nodes and in the smooth muscle of the walls of blood vessels. Very little was present in the numerous preparations from our recent case of human hemosiderosis. In the transfused rabbits a yellow pigment which failed to respond positively to the Nishimura test was observed in scattered connective tissue cells of the lungs and choroid plexus.

Comparison of the Human and Rabbit Organs.

Spleen.—The condition of the spleens of rabbits long transfused justifies the conclusion already expressed that this organ serves as a buffer depot for hemosiderin, and that its activity in this direction must be overcome if the pigment is to reach the other viscera in quantity. In animals killed after only 3 to 4 weeks of plethora the spleen was much enlarged, turgid with cellular debris and with phagocytes crowded with red cells. The liver and other viscera were at this period practically non-pigmented. But when the plethora had been maintained for months an entirely different state of affairs was found. The spleen, no larger than before, and often rather small, was a mere

[11] Nishimura, J., *Centr. allg. Path. u. path. Anat.*, 1910, xxi, 10.

congeries of sinuses, containing large, irregular masses of hemosiderin, and distended with blood, but almost empty of other cells (Fig. 1). The Malpighian corpuscles were atrophic, and many had nearly disappeared. The organ was, in fact, a mere shell, scarcely to be thought of as functioning longer for the retention of blood pigment. And, as would naturally follow from this fact, the liver and many other organs now showed profuse deposits of hemosiderin.

In the hemochromatosis of man there is chronic passive congestion of the spleen consequent on the liver cirrhosis characteristic of the disease. Phagocytes distended with hemosiderin are present in moderate number, but the greater part of the pigment, which at most is not abundant as compared with the amount in other organs, lies in the reticular cells. The Malpighian bodies are not atrophied. A diffuse increase in the connective tissue may usually be noted. There is no sign that a great activity of the organ to store up pigment was once present and has been overcome.

Bone Marrow.—The marrow of the rabbit, after months of plethora from transfusion, showed little more pigment than after a few weeks (Fig. 2). Evidently the limit of its capacity for the reception of hemosiderin had soon been reached. The greater part of the pigment lay in distended mononuclear cells. The entire quantity was only moderate. In man, too, this is the case. No distinct changes in the blood-forming tissues are to be noted in either animal.

Liver.—In the rabbits this was, after the spleen and certain of the abdominal lymph nodes, the most heavily pigmented organ. In one instance the degree of siderosis approached that in some patients dying of hemochromatosis (Fig. 3). The parenchyma contained everywhere numerous pigment granules but notably at the periphery of the lobules, where often the cells were filled with coarse yellow lumps that turned deep blue when tested by Nishimura's method. Similar iron-containing pigment lay in the capillaries, much of it in great rounded masses enclosed more or less completely in multinucleate giant cells. Many Kupffer cells were filled with pigment granules. Towards the center of the lobules the capillaries were widely distended, without doubt as a result of the plethora, and there was some pressure atrophy of the otherwise normal looking parenchymal elements. In two of the most pigmented livers there was some intralobular cirrhosis, but in a

third this element was quite lacking, and careful study has convinced us that such cirrhosis as was found was intercurrent in character, of a sort frequently seen in supposedly normal rabbits. The question of its relationship to the pigmentation will be discussed in detail further on. The cirrhotic tissue contained some new-formed bile ducts, but neither these nor the normal ones were pigmented, though here and there a connective tissue cell was laden with the characteristic brown granules.

In man the siderosis of the liver parenchyma has the same general appearance and an identical distribution with that in the rabbit, being more abundant towards the periphery of the lobules. Here many of the parenchymal cells may become so filled with pigment as to break down, resulting in connective tissue proliferation and the appearance of islands of new-formed liver cells. There is regularly present a pronounced hypertrophic cirrhosis with many new-formed and heavily pigmented bile ducts. This characteristic feature of the human disease was absolutely lacking in our rabbits.

Kidneys.—There was in the rabbits a marked siderosis of the kidney parenchyma (Fig. 4) somewhat more general in its distribution than is the case in human hemochromatosis. In this latter disease the pigment is, as a rule, sharply localized to the ascending limb of Henle's loop and to the distal convoluted tubules, where it is abundant; while the rest of the parenchyma, save in some cases the glomeruli, is free from it. Gaskell and his associates[9] point out that in pernicious anemia, by contrast, the pigment is found almost wholly in the proximal convoluted tubules. In our rabbits the glomeruli never showed pigment, but the cells of nearly all the cortical tubules contained it. Very fine granules were present in moderate number in the proximal convoluted tubules; more were to be seen in the descending limb of Henle's loop; and the ascending limb and distal convoluted tubules were very heavily pigmented with coarse, granular agglomerates, just as in the hemochromatosis of man. And, as in this disease, the collecting tubules and medulla were free from pigment.

Pancreas.—Only in the animals transfused during a long period was this organ pigmented. Fine hemosiderin granules were then demonstrable in the alveolar cells, often in considerable quantity (Fig. 5). The islands of Langerhans were always normal, and there was an en-

tire absence of the cirrhosis which in human beings may be marked. But there was an absence too of the destruction of gland tissue through excessive pigmentation which many authors hold to be the cause of the cirrhosis.

Adrenals, Stomach, and Intestines.—In two rabbits the adrenal glands showed a slight siderosis and in precisely the region where pigmentation is found in man; namely, in the zona glomerulosa of the cortex (Fig. 6). The intestines, as in the human instance, were free from hemosiderin save for the granules in a few connective tissue cells scattered throughout the coats. But the stomach also was unpigmented whereas in the human stomach the glands of the mucosa are usually the subject of an outspoken siderosis, especially at their base.

Heart.—This organ showed pigment only in the rabbits that were transfused longest. The hemosiderin was deposited in fine granules throughout the muscle fibers, being especially abundant close to either end of the nucleus (Fig. 7). An identical localization of the pigment is found in human beings, but the amount is, as a rule, far greater, sometimes leading to death of the muscle fibers with connective tissue overgrowth as a further result.

Lungs.—Neither in man nor in the rabbit is there a noteworthy pigmentation of the lungs. A few iron-containing cells may be present here and there in the reticulum and sometimes in the capillaries.

Lymph Nodes.—In the rabbits with most advanced siderosis large masses of free iron-containing pigment were present in the sinuses of the lymph nodes draining the liver (Fig. 8) and granules were present in many cells, as well. The other abdominal lymph nodes also contained iron in some quantity, but so sometimes do those of the normal rabbit. The mediastinal lymph glands of the transfused rabbits and those from the groins and axillæ showed at most only a few granules. This localization of hemosiderin to the nodes draining heavily pigmented organs is the rule in human hemochromatosis.

Skin.—The bronzed portions of the human skin usually contain slaty brown pigment granules in the basal layers of the epithelium, which do not react positively to the tests for iron. Ordinary hemosiderin is present as well in connective tissue cells of the corium, especially about the sweat glands. In our rabbits the epidermal pigment was lacking, but connective tissue cells containing hemosiderin were often fairly numerous in the corium (Fig. 9).

Other Organs.—In one of Sprunt's cases the tracheal cartilage was pigmented and in another the submaxillary gland. Both these lesions have been found in our rabbits. Siderosis of the submaxillary gland was well marked in one animal, the granules lying grouped in the alveolar cells (Fig. 10). The rabbit thyroid, on the other hand, was never affected, whereas in man it may be loaded with pigment. The rabbits transfused longest happened to be old females. In them a pronounced siderosis of the mammary gland was regularly present, certain cells of the epithelium being distended with pigment, while still others containing it had desquamated and could be found free in the lumen of the ducts (Fig. 11). There is no parallel in man for this lesion, since human hemochromatosis is a disease of the male. Abbott's description of the single case on record in a female does not include a report on the mammary gland.

Relation of Cirrhosis and Pigmentation.

Hypertrophic cirrhosis of the liver is a constant feature of human hemochromatosis, but whether it is a primary or secondary factor in the disease has not been determined. There is no doubt that a fibrous overgrowth is often present that has been induced by parenchymal destruction. The evidence of such a secondary connective tissue proliferation is so clearly visible in autopsy material that many authors have been led to consider the entire cirrhosis as secondary. But actually the recognition of areas of induced cirrhosis proves nothing either way, since they would be present in any event were the pigmentation sufficient to cause parenchymal destruction.

The transfused rabbits were killed at a relatively early stage of hepatic siderosis, and the ability of this latter to induce cirrhosis in the absence of cell destruction can be well judged from the findings. At the periphery of the lobules the parenchymal cells were heavily pigmented, but as yet only here and there had one broken down. Cirrhosis was practically absent, save for that of an intercurrent nature, which at most was slight, and obviously it had not followed the pigmentation, being often well marked where the latter was negligible. Here and there in the lobular capillaries were great masses of iron pigment, some of them as much as 80 μ across, more or less completely enclosed in multinucleate giant cells. But though these pigment

masses had induced pressure atrophy of the surrounding parenchymal cells, no connective tissue reaction had taken place about them. It may be urged that proliferation might have ensued in time, and this is not impossible. Yet in view of the lack of an immediate reaction the conclusion seems justified that hemosiderin when enclosed in living cells has no noteworthy stimulative effect on connective tissue.

Hepatic changes similar in degree to those in the rabbits cannot be expected at the average human autopsy, since patients with hemochromatosis do not, as a rule, succumb to the disease until the pigmentation has destroyed large numbers of parenchymal cells with a resulting connective tissue overgrowth. Only by some chance may one expect to obtain the liver at an earlier stage. Such a chance has recently come to us in the case of hemochromatosis already mentioned. The patient died, not of this disease, which apparently had lasted about $1\frac{1}{2}$ years, but of an old chronic myocarditis.[10] The cells at the periphery of the liver lobules were pigmented only to the degree found in rabbits and showed no evidence of injury. But here the parallel ceases. For whereas in the rabbits an hepatic cirrhosis was absent, in the human liver a strikingly abundant one was found, interlobular, and of long standing, as shown by its character. Save in the cells of the many new-formed bile ducts, the cirrhotic tissue contained almost no pigment. That the connective tissue overgrowth was in this instance the result of parenchymal destruction from excessive pigmentation is out of the question. One is led inevitably to conclude that it must have been a primary feature of the patient's disease.

If the fact is granted, as the evidence warrants, that cirrhosis is a primary occurrence in some cases at least of hemochromatosis, this does not mean that it is without a relation to the characteristic pigmentation. Kretz[12] has shown that cirrhosis of any sort is frequently accompanied by a more or less marked siderosis of the liver. He found such a pigmentation in fourteen out of twenty-six cirrhotic livers, whereas in only one of every fifteen or twenty that were non-cirrhotic was it present. The rabbit material has furnished striking proof that when a tendency to siderosis exists an intercurrent cirrhosis will

[12] Kretz, R., *Centr. allg. Path. u. path. Anat.*, 1897, viii, 620.

greatly increase the deposition of pigment. In one transfused animal with a very moderate general siderosis of the liver an adhesion had occurred connecting the anterior edge of a lobe with the abdominal wall and thus inducing a well marked local cirrhosis. Here the liver cells were so heavily pigmented as in many instances to be scarcely recognizable and in others to have broken down (Fig. 12). The contrast to the parenchyma elsewhere in the organ was extreme. In a second animal, as well, a local cirrhosis of unknown origin was accompanied by great local pigmentation. It is perhaps not a matter of accident that the rabbit with the most pigmented liver had not been transfused longest but was the subject of an intercurrent cirrhosis. In the course of 167 days it had received 139 transfusions—52 of 10 cc. each and the last 87 of 15 cc. each. A companion animal of the same weight and sex, receiving in 194 days 156 transfusions, the last 99 of 15 cc. each, had no cirrhosis of the liver and exhibited by contrast little pigmentation.

All this gives good reason for the supposition that the pigmentation of the liver cells in human hemochromatosis is in large part, if not entirely, secondary to the cirrhosis. Whether the connective tissue overgrowth of the pancreas that is so often observed is an essential phenomenon of the human disease or is secondary to the abundant parenchymal destruction, our work does not enable us to say. But a similar fibrous overgrowth, which is obviously of secondary character, may often be observed in the heavily pigmented heart of hemochromatosis cases, and sometimes in the thyroid gland.

Injury and Pigmentation.

Signs of an old perirenal inflammation existed in one of the transfused rabbits, and in the scar tissue here numerous connective tissue cells were noted containing hemosiderin. The observation led us to inject agar into the other animals of the series some days before killing them, with a view to determining whether iron would be laid down in the reactive tissue. Such was the case. The cells in the interior of the agar mass were unpigmented, which was to have been expected, since they were in process of rapid proliferation, but in the older reactive tissue hemosiderin was fairly abundant as small granules in the fibro-

blasts (Fig. 13). Its constant presence and general distribution rules out the possibility that it was derived from local hemorrhages.

A similar relation of injury to pigmentation was observed in the human instance of hemochromatosis already several times cited. The arteries showed scleroses here and there. In the sclerotic patches iron was sometimes encountered in quantity (Fig. 14), whereas in the healthy arterial wall it was entirely lacking. The ability of hepatic cirrhosis to induce pigmentation is, of course, only another example of the effect of injury, but of injury this time to an organ essentially concerned in the degradation of blood pigment.

A simple explanation now suggests itself for the curious distribution of the cutaneous pigmentation in human hemochromatosis. The face and neck to the collar line, and the backs of the hands are the surfaces which become most bronzed and are often the only ones affected. These are precisely the surfaces most subjected to light, wind, and other injurious influences, such as may be supposed from the evidence just given to conduce to a deposition of pigment. There is, however, no gainsaying the fact that not every sort of injury can bring about cutaneous siderosis, else one would expect it on the soles of the feet, for example. But the data given by Kretz show that even in the liver the injury must be of a special sort if iron is to be laid down. He has pointed out that neither the liver degenerations which result from phosphorus and arsenic poisoning, nor chronic passive congestion, nor cholelithiasis with jaundice suffices to cause the least hepatic siderosis.

An Aid to Diagnosis.

Hemosiderin granules were frequently found lying free in the lumen of the kidney tubules of the heavily pigmented rabbits, and they were also noted embedded in casts (Fig. 4). The fact suggested that in human beings a diagnosis might be made through the demonstration of hemosiderin granules in the urine. This would be far preferable to the excision of a piece of skin, the procedure usual at present. A diagnosis has been accomplished by the method, as is shown in another paper.[10]

On the Sequence of Events in Human Hemochromatosis.

The hemochromatosis of transfused rabbits, as thus far studied, has points of difference from the human disease, but also important points in common with it. The points of difference are in large part those of degree and of time. For instance, none of the animals developed diabetes; but for that matter in none was the pancreas greatly damaged as it is in *diabète bronzé*. The pigmentation of the pancreas, as far as it went, was like that occurring in man. This example well illustrates the general variances and likenesses found. The pigmentation of the rabbits was strikingly similar to that in human beings, not merely in kind but in distribution to the organs and in them. The sole difference in the pathological findings which must be considered as essential was the total absence in rabbits of the hepatic cirrhosis which in man is a constant and pronounced feature of hemochromatosis. There are good reasons, already stated, for the belief that this cirrhosis is a primary factor in the disease.

Most of the recent evidence in the literature is against the older view of excessive blood destruction in an otherwise healthy organism as the cause of hemochromatosis. So too are our experiments with rabbits. The transfused animals had disposed of immense quantities of blood before any noteworthy pigmentation appeared. Some of them received in the course of each week during a long period an amount of blood almost as great as their own original total quantity (5.5 per cent of the body weight).[13] The organism was required to dispose of this and of its own worn out corpuscles as well, yet at most the pigmentation was moderate compared with that found in man. Needless to say this excessive and continued blood destruction could not be supported by the human body without notable changes in the circulating red cells and the blood-forming organs, and these are completely absent.

While an increased destruction of red cells cannot be the primary cause of hemochromatosis, yet certainly one need look no further than these elements for the source of the hemosiderin. The presence of the pigment in such peculiar situations as the heart muscle and the parenchyma of many glands has led some authors to suppose that there must

[13] Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1909, xiii, 256.

be a derangement of these tissues, causing them to elaborate hemosiderin out of their own proper substance. But in our rabbits the pigment laid down was manifestly attributable to erythrocytes undergoing destruction, and it was found in precisely the same situations as in human hemochromatosis.

The activity of the liver is known to be profoundly important to the working over of blood pigment. The sequence of events in human hemochromatosis would seem to be the following: A liver cirrhosis of unknown origin leads to a failure of the organ to deal adequately with the iron-containing products of normal blood destruction, and the latter accumulate in the organism. Widespread pigmentation results automatically. It at length becomes so great as to cause the death of parenchymal cells, especially in the liver and pancreas, thus leading to secondary connective tissue overgrowth. This, in turn, through injury to the remaining parenchyma, hastens the accumulation of pigmentation. By a repetition of the vicious circle what is essentially a chronic disease becomes at length a rapid progress down-hill. Diabetes appears in many cases as a result of the pancreatic injury, and soon death results. There is no link in this chain of events for which the facts do not afford strong evidence.

SUMMARY.

In rabbits destroying transfused blood constantly during a period of many months a pronounced and widespread siderosis ensues, practically identical with that characterizing human hemochromatosis. The findings do not indicate the ultimate cause of this disease, but they throw light on its various features and its course, and suggest a means for its diagnosis.

EXPLANATION OF PLATES.

The sections from which Figs. 1 and 8 are taken were stained with methylene blue and eosin. Nishimura's method was used with all the other material to demonstrate iron-containing pigment, and lithium carmine was employed as a counterstain.

PLATE 64.

FIG. 1. Spleen of a rabbit (A) transfused 139 times during a period of 167 days. The sinuses are distended with blood. An atrophic Malpighian corpuscle may be

seen near the center of the photograph. The black masses consist of hemosiderin, for the most part extracellular.

FIG. 2. Bone marrow of the same animal. The hemosiderin appears black, as is the case also in the photographs which follow.

PLATE 65.

FIG. 3. Liver of the same animal as in Figs. 1 and 2. The cirrhosis is intercurrent. The iron is especially abundant towards the periphery of the lobules, but the parenchyma shows granules of it everywhere.

FIG. 4. Kidney of a rabbit (B) transfused 120 times in the course of 180 days. The glomeruli are free from pigment, but it is present in all the tubules except the collecting ones, being especially abundant in the ascending limb of Henle's loop and in the distal convolutions. A cast is to be seen containing many fine hemosiderin granules.

PLATE 66.

FIG. 5. Pancreas of a rabbit (C) transfused 156 times in the course of 194 days. The alveolar cells towards the center of the photograph contain hemosiderin in especially large amount.

FIG. 6. Iron-containing pigment in the peripheral cells of the zona glomerulosa of the adrenal gland. Rabbit C. The connective tissue capsule of the organ occupies the upper part of the photograph.

PLATE 67.

FIG. 7. Heart muscle of Rabbit A. The hemosiderin is especially abundant at either end of the nuclei of the muscle fibers.

FIG. 8. Section of a lymph gland draining the liver of Rabbit A. Large aggregates of extracellular hemosiderin are to be seen.

PLATE 68.

FIG. 9. Skin from the abdomen of Rabbit B. Iron-containing pigment is present in scattered cells of the corium.

FIG. 10. Hemosiderin granules in the alveolar cells of a submaxillary gland. Rabbit C. The patchy localization here seen was present throughout the gland.

PLATE 69.

FIG. 11. Mammary gland of Rabbit A, an old female. Many of the heavily pigmented cells have desquamated. The animal had not been pregnant during the period of transfusion.

FIG. 12. Heavy pigmentation secondary to a local cirrhosis of the liver. Rabbit B. Non-cirrhotic tissue may be seen at the lower left hand corner of the photograph. Here the pigmentation of the parenchyma is by contrast very slight.

PLATE 70.

FIG. 13. Hemosiderin in cells of the reactive tissue surrounding a mass of agar-agar. Rabbit B. The clear material is the agar, which is in process of organization. Iron-containing pigment is present only in the older scar tissue, not where the cells are actively proliferating.

FIG. 14. Sagittal section of a small artery from the patient with hemochromatosis. Iron-containing pigment has been deposited where the media is degenerated. The rest of the vessel wall is free from it.

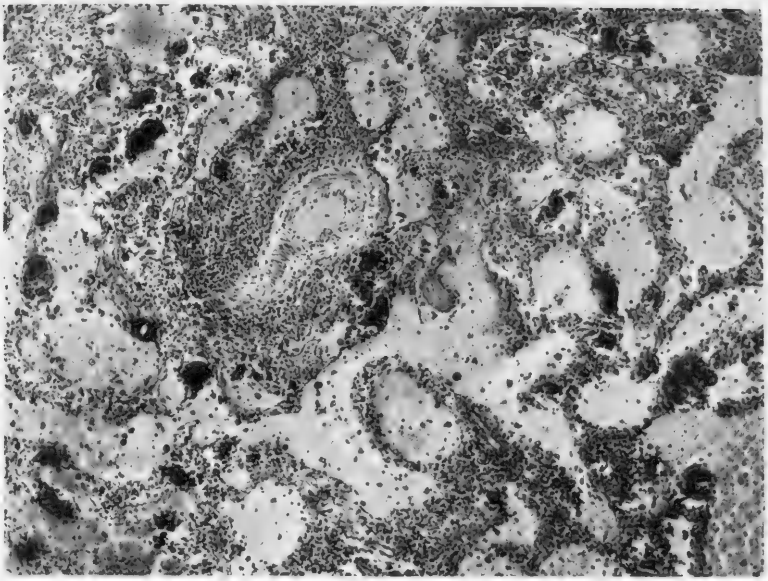

FIG. 1.

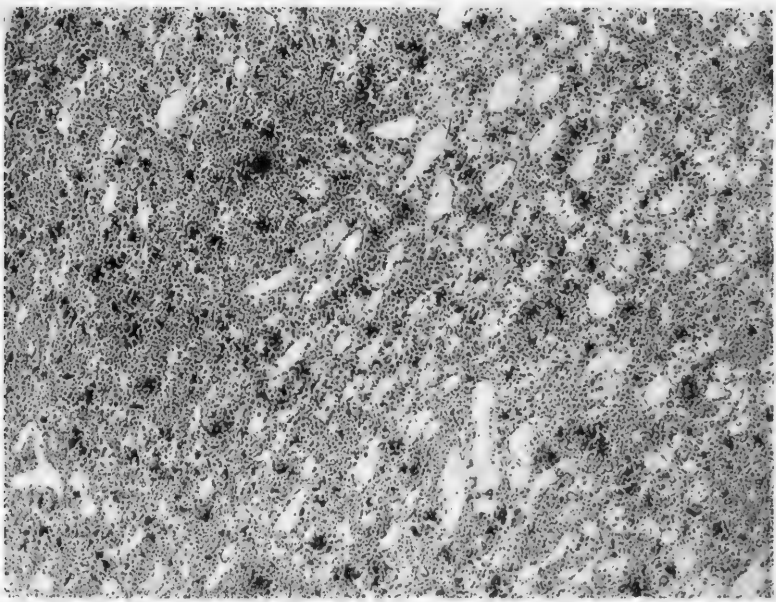

FIG. 2.

(Rous and Oliver: Experimental hemochromatosis.)

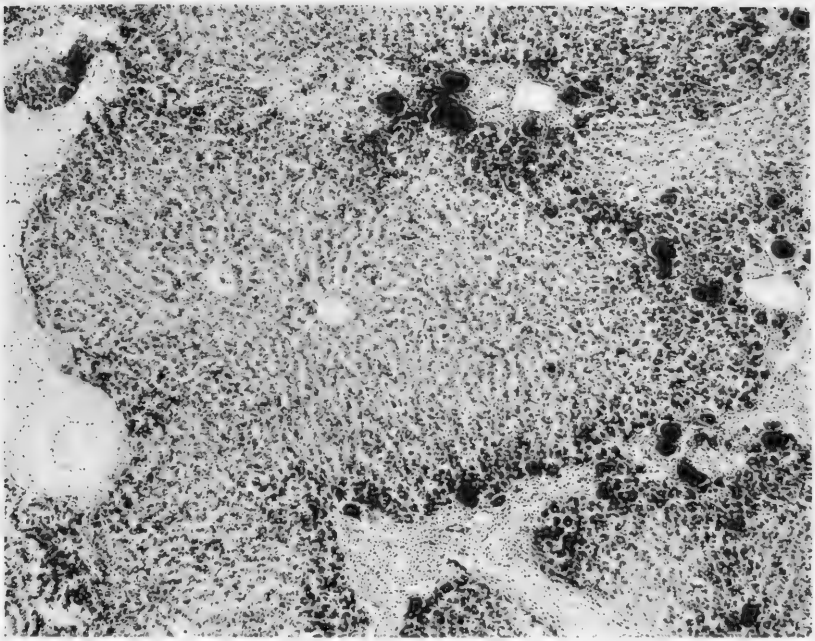

FIG. 3.

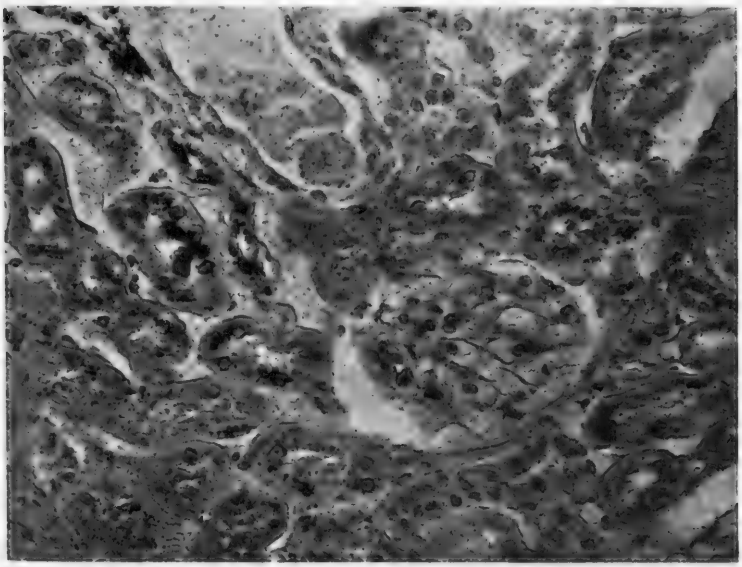

FIG. 4.

(Rous and Oliver: Experimental hemochromatosis.)

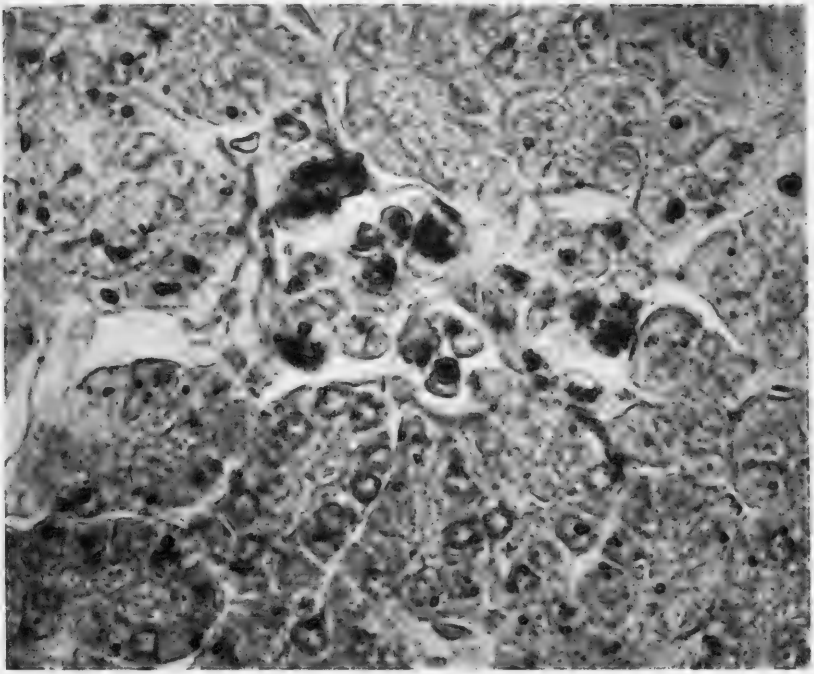

FIG. 5.

FIG. 6.

(Rous and Oliver: Experimental hemochromatosis.)

FIG. 7.

FIG. 8.

(Rous and Oliver: Experimental hemochromatosis.)

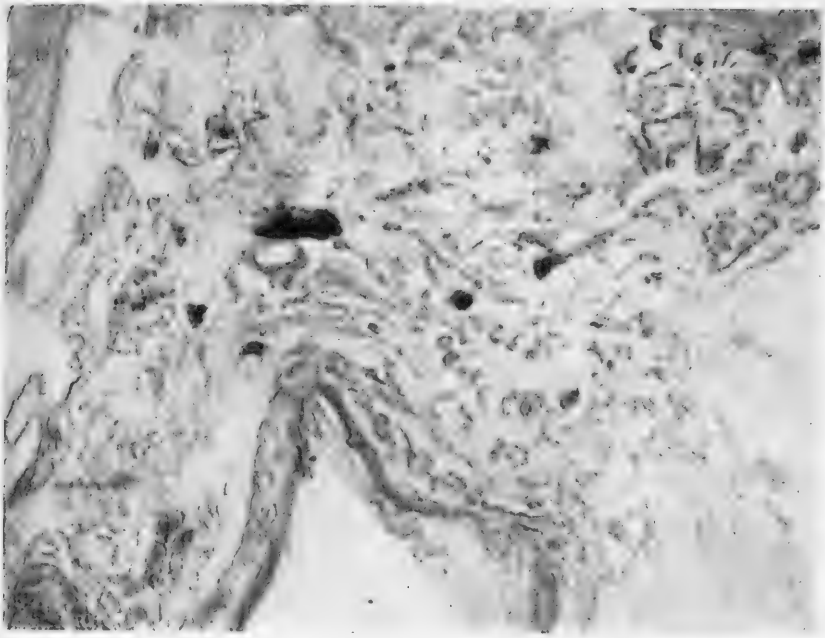

FIG. 9.

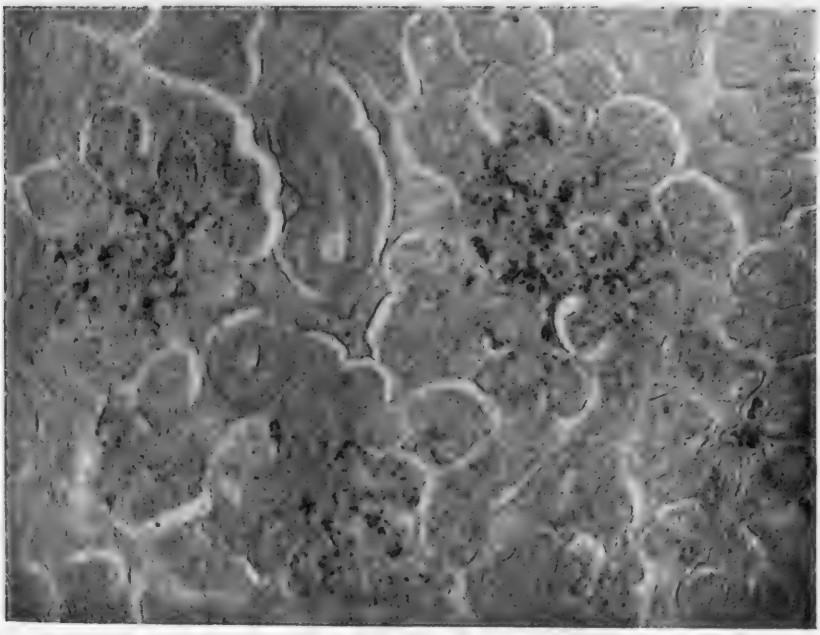

FIG. 10.

(Rous and Oliver: Experimental hemochromatosis.)

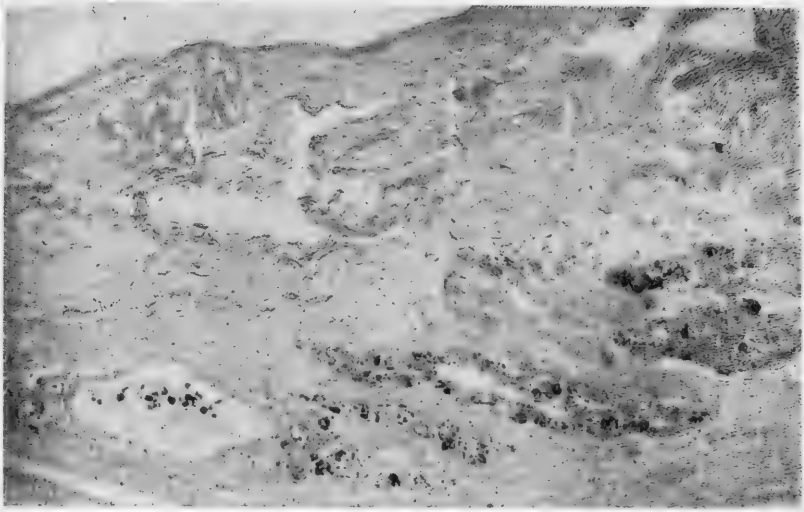

FIG. 11.

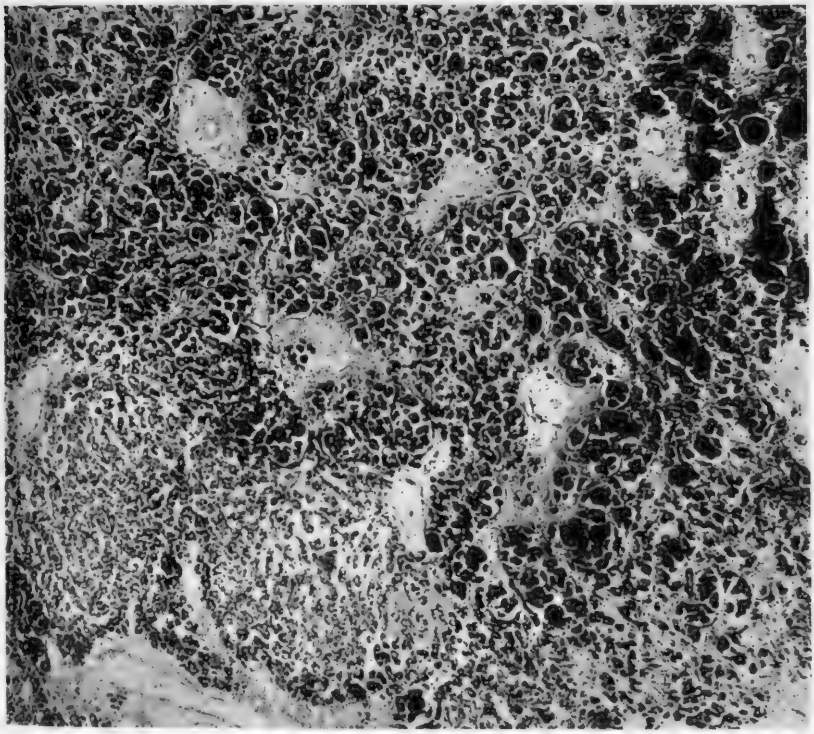

FIG. 12.

(Rous and Oliver: Experimental hemochromatosis.)

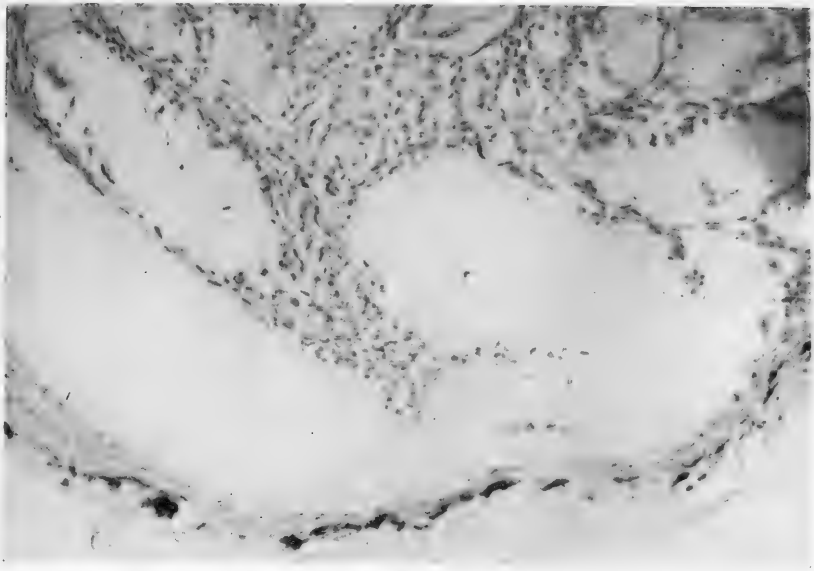

FIG. 13.

FIG. 14.

(Rous and Oliver: Experimental hemochromatosis.)

URINARY SIDEROSIS.

Hemosiderin Granules in the Urine as an Aid in the Diagnosis of Pernicious Anemia, Hemochromatosis, and Other Diseases Causing Siderosis of the Kidney.

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Plates 71 to 76.

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The siderosis produced in rabbits by transfusions repeated over a long period[1] has as one of its features a pronounced pigmentation of the kidney parenchyma comparable with that encountered in the hemochromatosis of human beings. Great numbers of yellow-brown granules which react positively for iron are present in the cells of the convoluted tubules and the loops of Henle. They are also to be found lying free in the lumen of the tubules, and embedded in casts. This fact suggested a search for hemosiderin granules in the urine of patients with pigmentation of doubtful origin. A test case soon came to hand.

A soldier, aged 46, was admitted to the Hospital of The Rockefeller Institute November 22, 1917. He complained of weakness, loss of weight, sugar in the urine, and swelling of the legs. One brother had died of diabetes at the age of 49. The patient himself had always been strong and well, save for appendicitis relieved in 1900 by operation. A Neisser infection in 1898 was followed by a stricture which yielded to treatment. Lues was denied. The present illness may be said to have begun in 1908, when, following 3 days of hard physical exertion, with loss of sleep, there occurred a sudden edema of the legs. The patient was in bed only a week, and the trouble did not reappear.

In December, 1916, the patient's friends noted that his skin was turning gray. He consulted a physician, who found sugar in the urine. Fasting, followed by a low diet, caused the sugar to disappear; and the patient's weight and strength, which had somewhat diminished, were restored. He entered the army service in

[1] Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

July, 1917, feeling well at that time, but soon began to have swelling of the feet and legs and shortness of breath. The division surgeon found sugar in the urine.

When admitted to the Hospital for treatment by Dr. Allen, the patient was not very ill. He was lean rather than emaciated. The skin of the face and of the back of the hands was somewhat freckled and looked weather-beaten, but more notable was a uniform, light, dusky gray pigmentation of these parts. The remaining body surfaces had no unusual tint, and the mucous membrane of the mouth was normal looking.

Physical examination disclosed a myocarditis, an enlarged, firm liver, ascites, and well marked edema of the feet and legs. The arteries were thickened. The liver edge was 9 cm. below the costal margin in the mm. line, very sharp and firm, and the surface of the organ was smooth, hard, and somewhat bulging. The spleen could not be felt. There was a well marked scar on the glans penis, but the Wassermann reaction was negative. The knee jerks were absent. The urine showed a large amount of sugar, but the ferric chloride test was negative. No albumin or casts were found.

The blood pressure was very low and so remained throughout the several months of hospital observation, ranging between 80 and 104 mm. systolic, and 46 to 70 mm. diastolic. There was a slight anemia: a hemoglobin averaging 85 per cent Sahli, with 4,400,000 red blood corpuscles per c.mm. Reticulated cells were rare on the one examination made, averaging 1 per thousand.

The diagnosis lay between syphilis, favored by the venereal history, genital scar, thickened vessels, absence of the knee jerks, and perhaps also by the enlarged liver, and the diabetes; Addison's disease, as indicated by the pigmentation, low blood pressure, and asthenia; and hemochromatosis, suggested by the combination of enlarged liver, pigmentation, and diabetes. Addison's disease was judged to be most likely.

Urinary Findings.

A fresh specimen of the urine was centrifugated and the slight sediment examined for hemosiderin. It consisted almost entirely of large cells containing numerous coarse, yellow-brown granules (Figs. 1 and 2). Often these granules were as big as red corpuscles, and had then the appearance of having been formed out of several smaller lumps. They were solid looking, moderately refractile, and of irregular shape, in distinction from the rounded, smooth globules of greenish yellow fat, sometimes met with them. Many of the cells were breaking down, and numerous free granules and larger lumps of the pigment were noted. All, both free and intracellular, turned a brilliant black

when submitted to ammonium sulfide, blue with potassium ferrocyanide and hydrochloric acid, and blue too in fixed specimens treated according to Nishimura's method.[2] There could be no doubt that they were composed of hemosiderin. The urine contained no blood, bile, urobilin, albumin, or casts.

The examination of the urine was repeated many times during the next 3 months. The output of hemosiderin granules was regularly abundant, and cells heavily pigmented with them were always obtained in large number from 20 cc. of urine. Control specimens from forty-four patients variously ill were searched for iron by the same methods. With one exception, which may be attributed to artefact, intracellular hemosiderin was found only in diseases involving, like *diabète bronzé*, a true siderosis of the kidney parenchyma. The diseases referred to are pernicious anemia and chronic hemolytic jaundice. In view of these results, the diagnosis of hemochromatosis seemed warranted in the case just described.

Autopsy Findings.

The patient died on April 3, 1918, 3 months after hemosiderin had first been recognized in the urine. Treatment had caused the urinary sugar to disappear promptly and permanently, but hyperglycemia had persisted. There was a gradually increasing edema of the legs and scrotum, with ascites and double hydrothorax; and death ensued from cardiac insufficiency. In the last weeks of life the cutaneous pigmentation became slightly more marked.

An autopsy, 1½ hours after death, performed by the author, disclosed the characteristic lesions of hemochromatosis. The anatomical diagnosis was as follows:

Myocarditis; hemochromatosis with pronounced pigmentation of the liver, pancreas, heart, abdominal lymph nodes, spleen, kidneys, thyroid, mesentery, skin, and other organs; cirrhosis of the liver and pancreas; chronic passive congestion of the liver, spleen, and intestines; ascites; hydrothorax; anasarca; fresh infarct of the lung; old infarcts of the kidneys.

[2] Nishimura, J., *Centr. allg. Path. u. path. Anat.*, 1910, xxi, 10.

The condition of the kidneys has special interest. There were present a few scattered, wedge-shaped areas of local atrophy and connective tissue overgrowth, the result of old infarcts. The general siderosis, though pronounced, was limited to certain groups of cortical elements, notably the distal convoluted tubules (Fig. 3). Here the parenchymal cells were heavily laden with iron-containing pigment, and some had desquamated, while others had broken down *in situ*, liberating pigment granules into the lumen of the tubules (Fig. 4). The cells of the ascending portion of Henle's loop were also pigmented, but less so; and in many of the glomeruli were cells containing numerous fine, hemosiderin granules. The remainder of the kidney was non-pigmented, save for occasional free cells and granules in the lumen of the collecting tubules. The distribution in the organ of the hemosiderin was precisely that which Gaskell and his associates[3] have described and deem characteristic of hemosiderosis.

Method of Search for Hemosiderin.

The following routine method of search for hemosiderin in the urine has proved satisfactory.

A fresh specimen of the urine, preferably warm from the body, is centrifugated at high speed and the supernatant fluid poured off as completely as possible. The sediment is suspended in the trace of fluid that remains, and slide preparations are gone over microscopically for suggestive orange or brown granules, more particularly in the cells. A mechanical stage should be used and 10 minutes at least given to the search. As a rule the sediment from a 20 cc. specimen will yield a fair number of cells from the higher portions of the urinary tract, but often that from 60 to 100 cc. must be obtained. Occasionally, in urine allowed to cool prior to centrifugation, the nubecula is copious and prevents proper concentration of the formed elements. When this is the case, a specimen should be centrifugated as soon as voided. Kept urines which have become cloudy with urates may be cleared by warming. To search a heavy crystalline sediment, or one poor in cells, or containing only leukocytes and squamous epithelium is time wasted. Catheterized specimens are necessary from female patients, since hemosiderin may come into the urine from the genital tract.

The remainder of the sediment is suspended in a fresh mixture of 5 cc. each of 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid. After 10

[3] Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913–14, vii, 129.

minutes it is thrown down with the centrifuge, the fluid poured off as before, a drop of the sediment mixed on the slide with a drop of the hydrochloric acid solution, and a cover-slip put on. By this modification of Perl's reaction the cells are well preserved and are largely cleared, as are the granular casts. Often the hemosiderin will begin to turn blue as soon as the acid has been added, but, if not, the preparation should be looked at repeatedly in the course of the next half hour. Usually there is present a little foreign debris which turns blue, giving an indication of when a positive finding may be expected. As in the case of the fresh sediment at least 10 minutes of search should be given to the preparation.

The use of a more certain method to demonstrate iron, namely that of Nishimura,[2] is often necessary. It is well known that hemosiderin in the tissues frequently fails to yield Perl's reaction, or to turn black with ammonium sulfide. This is likewise true of urinary hemosiderin. In several pernicious anemia cases suggestive looking orange granules were seen in the fresh, urinary sediment which proved refractory to the ordinary tests but were promptly shown to be iron-containing by the Nishimura procedure. The examination of fresh sediment assumes in this relation considerable importance as indicating that the search for iron should be persevered in. On the other hand, a failure to find suggestive pigment in the fresh specimen does not rule out the presence of iron. The ferrocyanide test has several times disclosed intracellular iron granules so minute or so surrounded by fat globules as not to attract attention in the fresh specimen. By Nishimura's method such instances are found even more often.

For the Nishimura test the fresh sediment, as free as possible from urine, is mixed with a little human serum untinted with hemoglobin and thick films are made and dried. These are fixed by heat, placed in strong ammonium sulfide for 1 hour, washed briefly with water, and subjected for 20 minutes to a fresh mixture of 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid in equal parts. After another brief rinsing with water the preparations are stained in lithium carmine for a few minutes, differentiated in acid alcohol (1 per cent hydrochloric acid), and run rapidly through 95 per cent alcohol, absolute alcohol, xylol, and mounted in balsam. The acid alcohol differentiates the red of the carmine and turns the iron granules a deep blue. Its action should be carefully followed with the microscope, since if prolonged it will dissolve the iron, or at least cause the blue tint of the latter to run and fade. Nishimura carried out two separate differentiations with acid, the first immediately after the ferrocyanide in order to bring out the blue of the iron reaction. But under these circumstances the subsequent action of the acid alcohol tends to destroy the blue color. A single differentiation is more satisfactory.

By this method permanent mounts are obtained to be looked over at leisure. The iron granules stand out in deep blue against the general carmine tint. Less satisfactory preparations may be had by submitting the films to ammonium sulfide and staining thereafter with carmine. Such hemosiderin granules as may react with the sulfide will then appear black. They lose their color rapidly in acid alcohol, so differentiation of the carmine should not be attempted with it.

Specimens left with preservatives prior to examination have proved unsatisfactory. Toluene permits autolysis of the cells, and formalin renders hemosiderin refractory to the ferrocyanide test and at length dissolves it. We have repeatedly found specimens which were negative, preserved with formalin and known to contain iron. Acid also dissolves the pigment, and it need not be looked for in very acid urines save when freshly voided. In old ammoniacal specimens it is remarkably stable and may persist undissolved for several days.

Findings in Pernicious Anemia.

The urines were examined of ten undoubted cases of pernicious anemia. In an eleventh case no proper search for iron could be made because of the heavy sediment of pus corpuscles, etc., incident to a cystitis. In two instances no hemosiderin was found. Repeated search was made of specimens from one of these having the history of a year's illness and a moderate anemia (red blood corpuscles 3,160,000; hemoglobin 69 per cent (Palmer)). In the other case the disease had lasted 3 years, during which period seventeen transfusions had been given. A single specimen only was searched for iron.

Intracellular hemosiderin was found in the urines of all the eight remaining instances, though not always easily. In two a careful search was necessary of specimens stained by the Nishimura method. The cells containing iron were very few and the pigment granules minute (Figs. 5 and 6). In a third instance Perl's reaction, applied to the fresh sediment, disclosed fairly frequent cells containing many fine, blue granules, half obscured amid fat droplets. In the five remaining instances a surprisingly large amount of hemosiderin was found (Figs. 7, 8, and 9). It attracted attention at once in the unstained sediment. Multitudes of fine, brownish, or orange-yellow, granules were seen, grouped in cells, scattered in casts, and more particularly lying free in large numbers. Save in one case, they were much smaller than in the urine of the patient with hemochromatosis already described, as would naturally follow from the relative intensity of the siderosis in the two diseases. From one to fifty or more granules were present in a single cell. They were spherical and remarkably regular in size in different specimens from the same patient, being usually somewhat smaller than eosinophil granulations. Occasionally they had cohered into lumps. The cells containing them were irregularly globular or

cylindrical, slightly larger than leukocytes, as a rule, with a vesicular nucleus of moderate size. Often they were much degenerated and contained myelin or fat. In two cases the modified ferrocyanide test caused the entire sediment to turn bright blue to the naked eye. With the microscope the color was found to be localized in innumerable, sharply defined deep blue granules. In the other instances with abundant hemosiderin the response to the test was only partial, a few of the granules turning blue or green. But with the Nishimura method all of the pigment was shown to be iron-containing.

Hemosiderin was never found in the squamous epithelium or in tailed cells from the bladder. Part, at least, of what was free in the urine obviously came from disintegrating cells. Indeed, the presence in the fresh sediment of small aggregates of orange-yellow granules held together by a remnant of cytoplasm proved significant. In the casts the granules were ordinarily few and widely separated.

A few of the urines showing iron in quantity had the deep, smoky color and the general characters usual in severe pernicious anemia. Others were pale, free from albumin, and seemed normal. No definite relationship could be worked out between the output of pigment granules and the duration and severity of the disease. The fact that pernicious anemia may long exist unsuspected has not improbably much to do with this. The findings in the individual case were remarkably constant from day to day. In one very sick patient, followed through $2\frac{1}{2}$ months, the hemosiderin output remained abundant. This was the exceptional case above mentioned, in which the granules were almost as large and abundant as in the patient with hemochromatosis.

Findings in Hemolytic Jaundice.

Through the courtesy of Dr. George Minot of Boston it was possible to examine the urine of a carefully studied, typical case of hemolytic jaundice of the acquired type. The patient, a male, aged 28, had been more or less jaundiced since the age of 16, following what he understood was an attack of malaria with enlarged spleen. The spleen is still greatly enlarged, but the jaundice is slight, the health excellent, and the blood in good condition. At the time when the urine was searched for iron the blood showed a hemoglobin of 102 per cent Sahli,

but the red cells were rather unequal in size, with many macrocytes and about 6 per cent of reticulated corpuscles. In the urine free hemosiderin was present in quantity, and there were numerous cells stippled with it. Most of the pigment responded readily to Perl's test. The intracellular granules were small, about the size of those usual in pernicious anemia, but much of the free pigment was in coarse, brown lumps. A cast was seen so colored with these as to resemble a miniature cigar.

Findings in Control Cases.

In none of thirty-three control cases, many of them with blood derangements, were such findings as the above obtained. In the fresh urinary sediment the numerous rounded, free, orange or brown granules were lacking, save in one notable instance to be discussed further on, and clumps of them held together by disintegrating cytoplasm were never observed. Pigmented cells, on the other hand, were fairly common. Often they were tinted a diffuse yellow or contained many fine, superimposed, greenish yellow fat droplets. Large yellow or orange-brown globules were also encountered sometimes. In general they were of a fatty or lipoid nature as indicated by their high refractility, smooth outline, resistance to acid, variation in size; and as proved finally by the Scharlach R stain. But occasionally intracellular granules were seen, indistinguishable in the fresh from hemosiderin but negative to microchemical tests. According to Aschoff,[4] an orange pigment, which fails to react for iron, is often present in the kidney cells of old people. A very fine crystalline deposit, furthermore, may simulate hemosiderin granules, free and in casts. Siderosis of the urine cannot be recognized with certainty from the fresh specimen alone. Microchemical tests must always be employed. In this connection it has been interesting to discover that Hunter[5] in 1889 published a note on a granular pigmentation of the cells and casts in the urine of a patient with pernicious anemia. From his description there is no reason to doubt that he had to do with a true siderosis, as was his own conviction. But he made no attempt to demonstrate iron by microchemical tests or to examine the urine of controls. His observa-

[4] Aschoff, L., Pathologische Anatomie, Jena, 2nd edition, 1911, ii, 431.
[5] Hunter, W., *Practitioner*, 1889, xliii, 321.

tion has never been followed up, probably because orange and brown granules are so frequently encountered in urines taken at random.

In one control urine a single cell was found containing iron in the form of several coarse lumps which gave Perl's reaction readily. The urine was an uncatheterized specimen from a woman with a poorly compensated cardiac lesion. No free hemosiderin granules were seen, and a second specimen carefully searched was entirely negative, as was the urine of several other heart cases. In view of the character of the granules and the general circumstances of the case it seems probable that the cell came from the genital tract. The finding emphasizes the necessity for catheterized specimens from female patients.

In another control instance much free hemosiderin was present in the urinary sediment but none in the cells. The patient had a chronic nephritis in the terminal stage with extreme anemia and constant slight bleeding from the mucous surfaces. The urine, of pale straw color, gave a weakly positive guaiac reaction, and a small number of red cells were present in the sediment. This latter contained numerous aggregates of very fine, rounded, slaty brown granules, which reacted positively to the tests for iron. In a second and catheterized specimen obtained 2 days later no red cells or shadows were seen, and there was only the faintest blue coloration on test with guaiac, yet the clusters, or aggregates, of hemosiderin granules were still present. The most careful search failed to reveal any cells containing such granules.

Some of the control cases deserve special mention. Three had the diagnosis of pernicious anemia on admission to hospital treatment, at which time the urine was examined. All had an extremely severe anemia, necessitating transfusion, a high color index, and in two instances a profusion of nucleated red cells were in circulation. One of the cases, that of chronic nephritis, has just been discussed. The other two, with urines negative for both free and intracellular hemosiderin, proved on study to be instances of secondary anemia, one subsequent to a severe purpura, the other associated with chronic malnutrition. The urinary findings were negative also in a patient with chronic polycythemia, and in two instances of Hodgkin's disease with great, progressive anemia.

DISCUSSION.

The presence of hemosiderin granules in the urine seems to have been overlooked heretofore. It is advisable to make a sharp distinction between the intracellular and free pigment, since there is probably a difference in their significance. True, when the granules are found inside urinary cells, free granules are regularly present also, and usually in far greater number. But free hemosiderin has been found alone in the urine of a nephritic patient whose condition presumably did not involve siderosis of the renal cells. Furthermore, extraneous material may easily be mistaken for free hemosiderin by the inexpert. Bits of foreign detritus frequently respond to the test for iron. As a rule, they are coarse and very different in appearance from the finely granular, true pigment.

The free hemosiderin granules occurring in the urine of patients with a true siderosis of the kidney are obviously of intracellular derivation as a rule. The granules have the size and appearance of those in the accompanying cells, and often a remnant of cytoplasm is attached to them or holds together a number of them in its matrix. The rest lie scattered broadcast in the sediment. In one instance, though, the free hemosiderin was of very different character, resembling that in the urine of the nephritic above mentioned. The granules, which were extremely minute, formed aggregates or clusters, sometimes of many hundreds, lying here and there in the urinary sediment (Fig. 10). Intracellular granules were rare. The findings varied little from day to day. The patient, a woman with severe pernicious anemia, had not been transfused, and there was no blood in the urine (guaiac reaction). A possible origin of the pigment from the genital tract was ruled out by catheterization. The inference would seem to be that in this case, as also in the nephritic, hemosiderin was circulating in solution within the body and was directly excreted by the kidney. This assumption is contrary to the general view, according to which during life hemosiderin arises only in cells, and as a granular solid limited to them or their neighborhood.[6] The problem presented is now under investigation in animals.

[6] Brown, W. H., (*J. Exp. Med.*, 1910, xii, 623) has demonstrated that free hemosiderin may be formed in the blood vessels of the autolyzing liver.

The evidence seems convincing that the presence of cells containing hemosiderin in urine obtained under proper precautions is the expression of an actual siderosis of the kidney parenchyma. These cells may be found in most instances, if not all, of diseases that involve such a condition. Fortunately the diseases are few and are readily distinguished from one another, whereas those with which they are oftenest confused produce no renal siderosis. The presence of intracellular hemosiderin in the urine assumes in this light importance for diagnosis.

On *a priori* grounds one may predict with some confidence that the examination of urinary cells for iron pigment will prove to be a decisive method of determining the presence of hemochromatosis in doubtful clinical instances of cutaneous pigmentation. For in hemochromatosis the siderosis of the skin is far surpassed by that of the internal organs. In hemolytic jaundice, congenital or acquired, the history, clinical features, and condition of the blood amply suffice for diagnosis; and the presence or absence of hemosiderin in the urine has interest merely as indicating the condition of the internal organs. In some of these jaundice cases no siderosis of the kidney is found at autopsy.[7] The exact bearing of the urinary findings on the diagnosis of pernicious anemia will require many observations. Positive findings cannot be expected in early stages of the disease before siderosis is established. But do cases come into the physician's hands then? Observations on the urine will throw light upon the point. This much, at least, is already certain, that a positive finding in the urine has significance for the diagnosis of pernicious anemia, whereas a negative one may have none. Even in advanced cases of the disease, as the present work has shown, cells containing hemosiderin may be so rare as to require, by the rather crude methods described, a persevering search for their discovery. Iron pigment is known to be relatively stable, once it has been deposited in the tissues. But whether it will persist sufficiently in the kidney—and the urine—to be useful in diagnosis during the remissions of pernicious anemia remains to be seen.

Besides the diseases mentioned, certain local or general conditions, fortunately rare, may conceivably lead to a urinary siderosis. Severe, long continued hemolysis of any kind, as for example that from malaria,

[7] Guizzetti, P., *Beitr. path. Anat. u. allg. Path.*, 1912, lii, 15.

may be expected to do so. According to Aschoff[4] a patchy siderosis of the renal parenchyma sometimes results from local hemorrhages. Furthermore, according to the same authority, hemoglobin excreted into the tubules may be taken up again by the living cells and converted into hemosiderin. Urinary siderosis becomes a possibility, therefore, in paroxysmal hemoglobinuria, or after the transfusion of incompatible blood. Finally, hemorrhages into the mucosa of the urinary passages may perhaps lead to a desquamation of iron-containing cells.

All but two of the pernicious anemia cases examined in the course of the present work had been transfused, some of them many times. One of the two untransfused patients had much hemosiderin in the urine, and the other none. The influence of successful transfusion to induce of itself a renal siderosis must be considered, since in normal animals transfusions frequently repeated over a long period undoubtedly have this result.[1] But the findings in transfused human beings who were among the controls in the present work are in agreement with the results in animals in showing that much alien blood may be introduced into the body before any renal siderosis develops. The urines of these clinical controls, some of whom had been many times transfused, were uniformly negative for iron-containing pigment granules. In one case, that of a girl of 14 with purpura, nine transfusions, totalling 6,000 cc. of blood, had been given in the month prior to the urinary examinations. Yet the urine, searched on several occasions, proved completely free from hemosiderin. The physiological conditions are different in pernicious anemia. Here transfused blood is in the end just so much more material to be destroyed in the course of the disease, with a resulting increased retention of blood derivatives. That siderosis may be enhanced thereby can scarcely be doubted.

The circulating leukocytes in the case of hemochromatosis were, in general, free from hemosiderin. Several examinations were made, using thick films of washed leukocytes stained for iron by Nishimura's method. After long search one cell was found containing a few small, blue granules.

SUMMARY.

In diseases which bring about a siderosis of the kidney there are ordinarily present in the urinary sediment cells containing granules of hemosiderin, and often many free granules as well. The finding has proved useful in the diagnosis of hemochromatosis and will probably be of service in the recognition of pernicious anemia, and possibly some other diseases. But in this relation the fact should be emphasized that urinary siderosis, as one may term it, is the indication of a renal condition, not of a disease.

My special thanks are due to Dr. Walter W. Palmer, to Dr. George Minot of Boston, and to Dr. Edward Lindeman, for their kindness in providing the clinical material essential to the work.

EXPLANATION OF PLATES.

PLATE 71.

FIG. 1. Free and intracellular hemosiderin in the urinary sediment of a patient with hemochromatosis. Nishimura stain.

PLATE 72.

FIG. 2. Drawing of an actual field in a fresh preparation of the same sediment as in Fig. 1. The large size and irregular shape of the pigment granules should be noted.

PLATE 73.

FIGS. 3 and 4. Kidney of the patient with hemochromatosis. Nishimura stain. In Fig. 4 pigmented cells and free granules can be seen lying free in the lumen of a tubule.

PLATE 74.

FIGS. 5 and 6. Minute hemosiderin granules in the urinary cells of a case of pernicious anemia. Nishimura stain. × 1,060.

FIG. 10. Masses of free granular hemosiderin in a urine with scarcely any of the intracellular pigment. The specimen is from a case of pernicious anemia. Nishimura stain. × 1,000.

PLATE 75.

FIGS. 7 and 8. Hemosiderin, free and intracellular, in the sediment from a patient with pernicious anemia. The abundant pigment has the form of rounded granules. One of the cells of Fig. 7 is so crowded with it as to appear almost black. The amount of free pigment is noteworthy.

PLATE 76.

FIG. 9. Drawing to show the appearance of the hemosiderin in fresh preparations from a pernicious anemia case. The cells are taken from several fields.

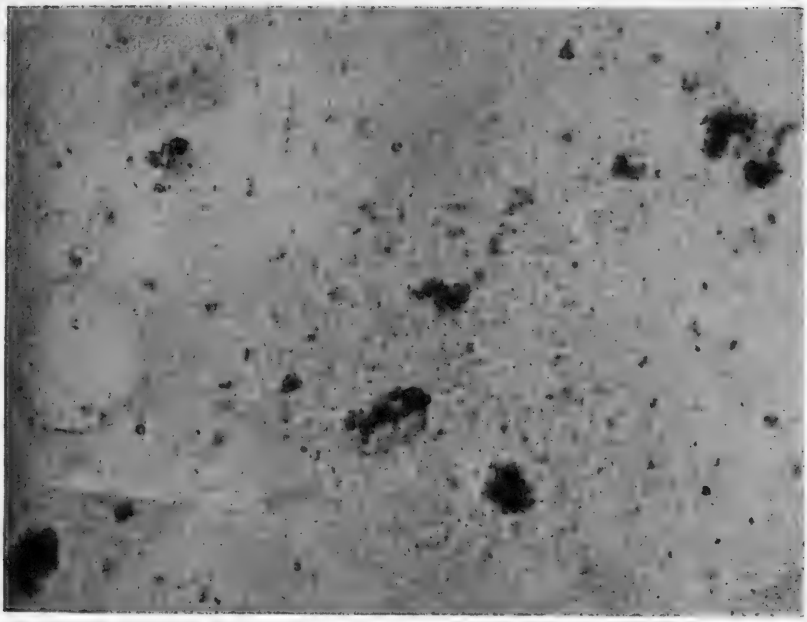

FIG. 1.

(Rous: Urinary siderosis.)

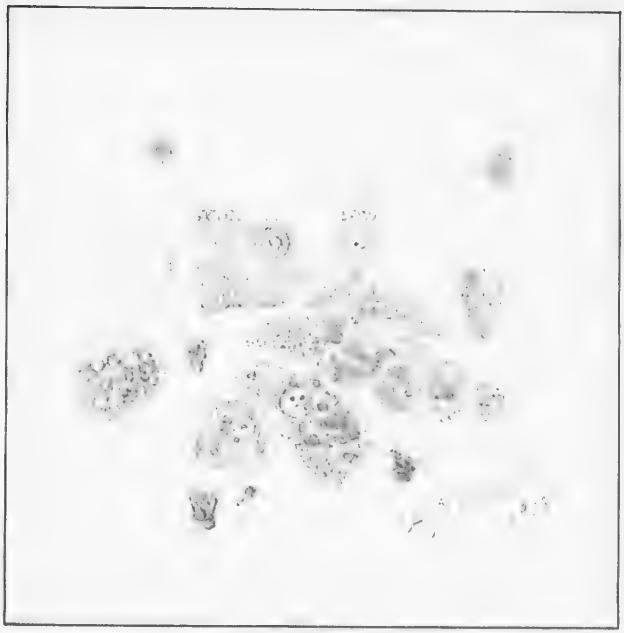

FIG. 2.

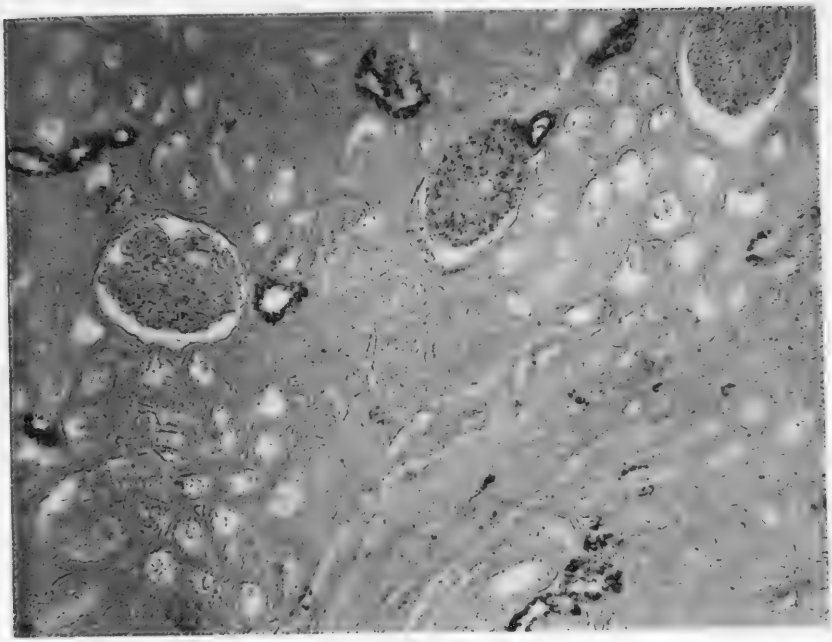

FIG. 3.

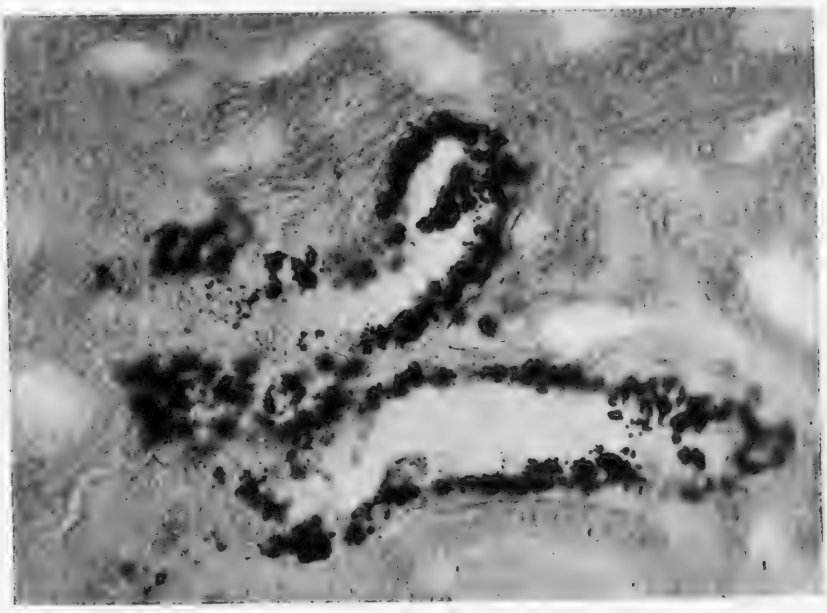

FIG. 4.

(Rous: Urinary siderosis.)

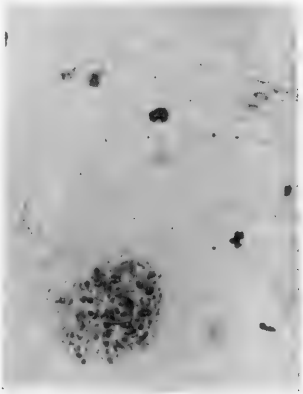

FIG. 5.

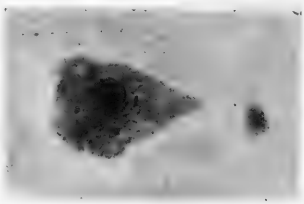

FIG. 6.

FIG. 10.

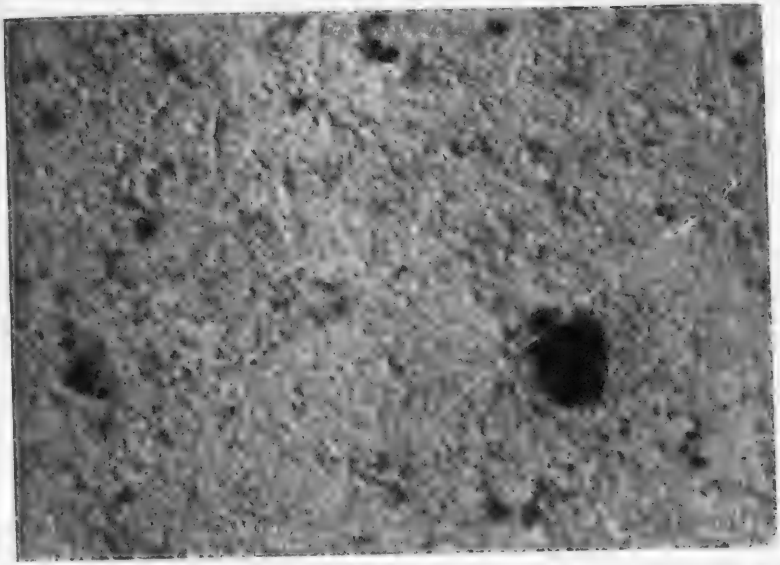

FIG. 7.

FIG. 8.

(Rous: Urinary siderosis.)

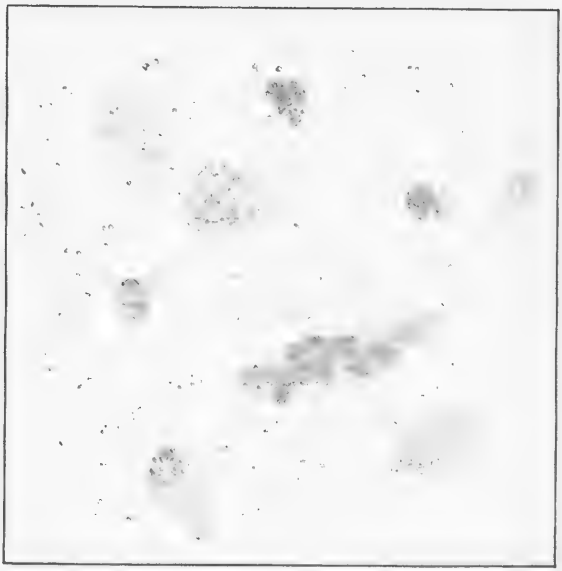

FIG. 9.

(Rous: Urinary siderosis.)

A RAPID DIFFERENTIAL METHOD FOR THE ISOLATION OF BACILLUS INFLUENZÆ.

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Plate 77.

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It has often been noted that on a blood agar plate seeded with influenzal sputum the colonies of *Bacillus influenzæ* are most numerous and grow to larger size in the neighborhood of colonies of certain other organisms, notably the staphylococcus. This phenomenon led the authors to conduct various experiments which may be summarized as follows: Blood agar plates were seeded uniformly with a pure culture of *Bacillus influenzæ* and were then streaked with hemolytic and non-hemolytic staphylococci, hemolytic and non-hemolytic streptococci, and hemolytic and non-hemolytic strains of *Micrococcus catarrhalis*. After incubation it was found that the more luxuriant growth of *Bacillus influenzæ* occurred only in the neighborhood of the streaks of hemolytic staphylococci, streptococci, and micrococci, not in the neighborhood of the non-hemolytic strains. On laked blood agar the colonies of *Bacillus influenzæ* grew well throughout the plate and were no more numerous in the vicinity of staphylococcus, streptococcus, or micrococcus streaks than elsewhere. It has been shown[1] that the well known zones of hemolysis produced by hemolytic streptococci in blood agar are largely if not entirely the result of mere laking of the blood corpuscles and outward diffusion of the released hemoglobin into the surrounding medium. We would conclude, therefore, that the more luxuriant growth of *Bacillus influenzæ* in the neighborhood of colonies of hemolytic cocci is the

[1] Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1918 (in press).

result of the presence of released hemoglobin diffusing outward from those colonies. The hemoglobinophilic habit of *Bacillus influenzæ* has long been recognized as one of its differential characters.

The phenomenon described above has been utilized by us for the isolation of *Bacillus influenzæ* from sputum. To tubes containing about 12 cc. of standard meat infusion agar, melted and cooled to

TEXT-FIG. 1. Glass rod used for spreading droplet of sputum over the blood agar plate.

45° to 50°C., is added 5 to 10 per cent of defibrinated blood. In the fluid condition this medium is inoculated with a small loop of suitably diluted washed sputum and is immediately poured into a Petri dish. When the blood agar has solidified a very small loop of undiluted washed sputum is deposited on the surface of the medium

and by means of the flamed glass rod shown in Text-fig. 1 the droplet of sputum is smeared uniformly over the surface. Without flaming, the rod is then similarly rubbed over the surface of a second blood agar plate on which no sputum has been deposited by the loop. The optimum distribution of colonies should thus be secured on one or the other of the two plates. After the plates have thus been inoculated with sputum, each of them is streaked, in the form of a single streak, circle, or cross, with a pure culture of markedly hemolytic staphylococcus or streptococcus. After the moisture which collects on the surface of the freshly poured agar has been allowed to evaporate, the plates are incubated and may be examined next day. *Bacillus influenzæ* grows in the form of minute discrete convex colonies without hemolysis or discoloration of the blood agar and often visible only by reflected light. If colonies of *Bacillus influenzæ* are present they will be found in largest numbers and of largest size in the vicinity of the zone of hemolysis produced by the streak of staphylococcus or streptococcus. Material from such colonies should be stained by Gram's method, an aqueous solution of safranine being a good counterstain for *Bacillus influenzæ*. This method enables one to isolate within 24 hours Gram-negative hemoglobinophilic bacilli which, if they resemble *Bacillus influenzæ* morphologically and if the material has come from a case of clinical influenza, may be regarded as *Bacillus influenzæ*. Horse, human, or rabbit blood may be used, but to be of differential value it should be in good condition, not laked.

In Fig. 1 is shown the photograph of a plate inoculated as described above. In this sputum the principal organisms were hemolytic streptococci, shown as deep hemolytic colonies scattered throughout the plate, and influenza bacilli. The plate was streaked in the form of a circle by a stock strain of hemolytic streptococcus. The small refractive colonies of *Bacillus influenzæ* (indicated by arrows) are seen to be very numerous within the zones of hemolysis produced by the deep colonies of streptococci, also in the neighborhood of the streak of stock streptococcus, and actually within the streak itself. By reflected light they could also be seen in smaller number and of smaller size in portions of the plate remote from the zones of hemolysis but these colonies cannot be seen in the photograph.

EXPLANATION OF PLATE 77.

FIG. 1. Blood agar plate inoculated deeply and over the entire surface with influenzal sputum, and then streaked in the form of a circle with a stock strain of hemolytic streptococcus. Small discrete colonies of *B. influenzæ* are indicated by arrows.

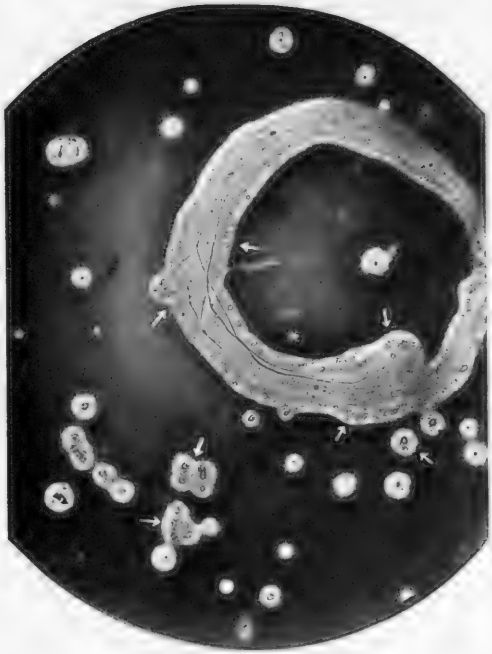

FIG. 1.

(Brown and Orcutt: Rapid method for isolation of *B. influenzæ*.)

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CONTENTS OF VOL. I, No. 1, SEPTEMBER 20, 1918

OSTERHOUT, W. J. V., and HAAS, A. R. C. On the dynamics of photosynthesis.

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UHLENHUTH, EDUARD. The antagonism between thymus and parathyroid glands.

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